RECOMBINANT HUMAN METAPNEUMOVIRUS AND ITS USE

PRIORITY CLAIM

This application claims the benefit of U.S. Provisional Patent Application No. 60/451,119,

5   filed February 28, 2003, and U.S. Provisional Patent Application No. 60/478,667, filed June 13,

2003. Both of the provisional applications are incorporated by reference herein in their entirety.

FIELD OF THE DISCLOSURE

10   This invention relates to the field of virology and, more specifically, to methods for

producing recombinat human metapneumovirus (rHMPV), for producing replication competent

derivatives with desirable properties such as attenuation and protective efficacy, and to uses of

rHMPV in immunoprophylaxis and therapy.

15   ## BACKGROUND

Human metapneumovirus (HMPV) is a virus that was first recovered in the Netherlands

from infants and children experiencing acute respiratory tract disease (van den Hoogen *et al*. Nat.

Med. 7:719-724, 2001; De Jong *et al*. WO 02/057/302 A2).

20   HMPV is worldwide in prevalence and resembles human respiratory syncytial virus (RSV or

HRSV) with regard to disease signs and the ability to infect and cause disease in the young infant as

well as individuals of all ages (for a review, see Heikkinen and Jarvinen, Lancet 361:51-59, 2003).

HMPV is characterized as an enveloped virus with a genome that is a single negative strand

of RNA of approximately 13 kb. The virus has been classified presumptively in the

25   Metapneumovirus genus, Pneumovirus subfamily, Paramyxovirus family of the Order

Mononegavirales, comprising the nonsegmented negative strand RNA viruses or mononegaviruses.

Mononegaviruses also are called nonsegmented negative strand RNA viruses. The Paramyxovirus

family has two subfamilies, Paramyxovirinae and Pneumovirinae (also referred to as

paramyxoviruses and pneumoviruses, respectively).

30   Several other mononegaviruses are important agents of respiratory tract disease in pediatric

and other populations, for example, RSV and human parainfluenza virus types 1, 2 and 3 (HPIV1,

HPIV2 and HPIV3). Although these viruses have some similarities with HMPV, their sequences are

different from the HMPV viruses disclosed herein.

Since HMPV was described only recently, there is little documented experience with its

35   propagation, manipulation and stability, and there are no well established or widely available

reference strains, mutant strains, reported vaccine candidates, or reference virus-specific antibodies or

comparable reagents or systems or experimental animals models to facilitate characterization.

Because HMPV is associated with severe respiratory tract disease, there is a need to develop methods
to engineer safe and effective vaccines to alleviate the serious health problems attributable to this
pathogen, particularly among young infants.

5   **BRIEF SUMMARY OF SPECIFIC EMBODIMENTS**

HMPV is a significant agent of human respiratory tract disease. Methods and compositions
are provided herein for recovering infectious, recombinant HMPV. Recombinant HMPV is disclosed
herein, including the complete nucleic acid sequence encoding all of the protein products of HMPV.
Compositions and methods for introducing defined, predetermined structural and phenotypic changes
10   into an infectious HMPV are disclosed, as are attenuated forms of HMPV as well as forms of HMPV
that have been modified to have improved qualities relevant to immunogenicity, safety, protective
efficacy, and breadth of vaccine coverage. Thus, methods are disclosed for generating an immune
response against HMPV in a subject. The methods include administering to the subject an attenuated
HMPV, thereby producing the immune response. Also described is the identification of a set of viral
15   ORFs whose expression is sufficient to direct viral transcription and RNA replication, and to produce
infectious HMPV entirely from cDNA.

The foregoing and other features and advantages will become more apparent from the
following detailed description of several embodiments, which proceeds with reference to the
accompanying figures.

20
                                     BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a diagrammatic representation of the negative sense genomes of HMPV strain
CAN97-83 (hereafter referred to as 83 or CAN-83) and RSV strain A2, showing a comparison of the
25   two genomes. Individual proposed genes are shown as shaded boxes separated by proposed
intergenic regions represented by open bars. The 3′ extragenic leader and 5′ extragenic trailer regions
also are shown as open boxes. Tentative HMPV gene assignments are based on ORFs whose
potential protein exhibits partial amino acid sequence identity or structural similarity with a known
protein from RSV. Note that HMPV lacks apparent counterparts to the NS1 and NS2 genes present
30   in RSV (boxed), and that the putative F and M2 gene pair precedes the SH-G gene pair in HMPV but
follows it in RSV (the difference in the position of SH-G is indicated with dotted lines). The M2 and
L genes overlap in RSV but may not do so in HMPV. In both viruses, the M2 gene contains two
ORFs M2-1 and M2-2, depicted as filled bars. Amino acid lengths are shown for the deduced
unmodified viral proteins. Each of the RSV proteins has been directly identified; none of the
35   proposed HMPV proteins had been directly identified prior to the current disclosure. Abbreviations
(with HMPV assignments by analogy to RSV): N, major nucleocapsid protein; P, nucleocapsid
phosphoprotein; M, inner virion matrix protein; M2-1 and M2-2, products of the first and second

ORFs, respectively, in the M2 mRNA; SH, small hydrophobic protein; G, heavily glycosylated
protein involved in attachment; F, fusion protein involved in penetration; L, large polymerase protein;
NS1 and NS2, nonstructural protein 1 and 2, respectively. This and all subsequent diagrams are not
to scale.

5   FIG. 2 is a diagram showing sequence alignments between the 3' leader (top) and 5' trailer
(bottom) regions and flanking areas of the genome of HMPV strain 83 (SEQ ID NO: 18 and SEQ ID
NO: 22, respectively), avian pneumovirus (APV or AMPV, SEQ ID NO: 19 and SEQ ID NO: 23,
respectively), and HRSV strain A2 (SEQ ID NO: 20 and SEQ ID NO: 24, respectively). The
sequences are in genome (negative) sense. Nucleotide assignments that are the same in two or more
10   sequences between HMPV 83, APV and RSV are shaded; sequence gaps introduced to make optimal
alignments are indicated by dashes. The bars over the left hand end of each sequence indicates
regions whose spacing and sequence had not been previously determined for any HMPV. The partial
sequences available for HMPV strain 00-1 3' leader and 5' trailer regions (SEQ ID NO: 17 and SEQ
ID NO: 21, respectively) are indicated at the top: nucleotide assignments in strain 00-1 that are
15   identical to those of HMPV 83 are indicated by asterisks while nucleotide differences are indicated.
Sequences: APV (Randhawa et al., J. Virol. 71:9849-54, 1997), RSV (Mink et al., Virology 185:615-
24, 1991), HMPV 00-1 (van den Hoogen et al., Nat. Med. 7:719-24, 2001).

         FIG. 3 is a diagram showing sequence alignments between the 3' and 5' ends of the genome
of HMPV strain 83 (top; bases 1-41 of SEQ ID NO: 18 and bases 1-40 of SEQ ID NO: 22,
20   respectively) and APV (bottom; bases 1-41 of SEQ ID NO: 19 and bases 1-42 of SEQ ID NO: 23,
respectively), with complementary nucleotides boxed. This shows that the last 13 nucleotides of the
two ends of APV are perfect complements, whereas HMPV unexpectedly has two noncomplementary
nucleotides. Sequences for HMPV that were not previously available are indicated with solid lines
above (leader, Le strand) or below (trailer, Tr strand) the sequence.

25   FIG. 4 is a diagram showing several examples of differences in nucleotide and amino acid
lengths between HMPV strains 83 and 00-1. The differences in G are illustrated by partial amino acid
sequences (upper alignment; amino acids 190-219 of SEQ ID NO: 6 and amino acids 190-236 of
SEQ ID NO: 11, respectively) and partial nucleotide sequences (second alignment; bases 6871-6929
of SEQ ID NO: 1 and bases 6886-6964 of SEQ ID NO: 36, respectively). The sequences are
30   numbered according to the amino acid sequence of HMPV 83 G (top alignment) or the complete
nucleotide sequence of the antigenome of HMPV 83 (second alignment). Examples of differences
include, (1) the SH protein for 83 is shorter by 4 aa due to an earlier stop codon; (2) the G protein for
83 is shorter by 17 aa due to an earlier stop codon, which is followed by a deletion in the downstream
noncoding region; and (3) the intergenic region between the F and M2 genes of 83 is 13 nucleotides
35   (nt) compared to 41 nt for 00-1. Taken together, these show that the G ORF and encoded G protein
of strain 83 are shorter by the equivalent of 17 amino acids from that of strain 00-1.

FIG. 5 is a chart showing the percent amino acid sequence identity for the predicted, putative proteins of HMPV 83 compared to those of two other strains of HMPV (00-1, and 97-82), three different antigenic types of APV (A, B and C), the two subgroups of human RSV (A and B), bovine RSV (BRSV), and pneumonia virus of mice (PVM, a murine counterpart of HRSV). Note that
5 HMPV is most closely related to APV C. ND indicates that a comparison was not done for the indicated subject.

FIG. 6 is a taxonomy tree, illustrating putative phylogenetic relationships between HMPV and other paramyxoviruses based on comparisons of the amino acid sequences of proposed matrix M proteins. Pneumovirinae and Paramyxovirinae are the two subfamilies of the Paramyxovirus family.
10 The other taxonomic names refer to genera. The numbers refer to the extent of amino acid sequence divergence (percentage non-identity) divided by 100; hence, 0.2 refers to 20% amino acid differences. Note that a system for recovering and modifying infectious recombinant virus has not been reported for any member of genus Metapneumovirus even though the avian members of this genus have been known for almost 25 years.

15 FIG. 7 is a diagram illustrating the construction of a cDNA encoding the complete antigenome of HMPV strain 83, designed from the complete consensus genomic sequence. Three separate subgenomic cloned fragments were created: fragment 1 contains the putative N, P and M genes and is bordered on the upstream (left) side by an added promoter for bacteriophage T7 RNA polymerase (T7p) and on the right hand side by an NheI site that was added to the putative M-F
20 intergenic region as a marker. The sequence changes involved in introducing the NheI site are shown. The T7 promoter was designed to add three nonviral G residues to the 5′ end of the antigenome, a configuration chosen to improve the efficiency of the T7 promoter. Fragment 2 included the putative F, M2, SH and G genes and is bordered on the upstream side by the added NheI site and on the downstream side by a naturally occurring Acc65I site. Fragment 3 consists of the L gene followed by
25 part of the hepatitis delta virus ribozyme (HDVribo) (Perrotta and Been, Nature 350:434-6, 1991) bordered by an RsrII site that occurs naturally within that ribozyme. The vector for cloning and expressing the HMPV 83 antigenome cDNA was pBSKSII, which is a derivative of a modified Bluescript II KS + (Stratagene) vector described previously (Durbin et al., Virology 71:4272-7, 1997) and contains the hepatitis delta virus ribozyme followed by a terminator for T7 RNA polymerase
30 (T7t). This vector was modified by the insertion of a polylinker containing AvaI, AatII, NheI, and Acc65I sites, which in turn served to accept the cloned fragments 1, 2 and 3. The final cDNA encodes the complete 13335-nucleotide HMPV 83 antigenome containing three added nonviral G residues at the 5′ end. The complete pHMPV-83 recombinant plasmid ("antigenome plasmid") contains 16333 bp.

35 FIG. 8 is a diagram showing putative transcription signals for HMPV strain 83 based on the identification of semi-conserved sequence motifs located between the major ORFs in the complete sequence. Sequences are in positive (mRNA) sense. The semi-conserved sequence motif that

precedes most of the ORFs in the complete sequence is shown as the putative gene start (GS) signal, with some of the most highly conserved sequences shown in upper case letters and with flanking sequence on either side in lower case. The individual GS motifs are named according to the putative ORF that each precedes (SEQ ID NO: 1, bases 29-64 for N; bases 1237-1272 for P; bases 2154-2189

5   for M; bases 3041-3076 for F; bases 4698-4733 for M2; bases 5455-5490 for SH; bases 6206-6241 for G; and bases 7107-7142 for L). A consensus sequence is shown underneath (SEQ ID NO: 25). For positions where alternative assignments can occur, these are listed below the consensus (SEQ ID NOs: 26 and 27). By this analysis, the major element of the GS signal is contained within sixteen nucleotides, of which positions 1, 3, 4, 6, 7, 9, and 14-16 were exactly conserved, with 14-16 also

10   serving as potential translation start sites. Similarly, the semi-conserved sequence motif that follows most of the ORFs in the complete sequence is shown as the putative gene end (GE) signal, with some of the most highly conserved sequences shown in upper case and flanking sequence in lower case. The individual GE motifs are named according to the ORF that each follows (SEQ ID NO: 1, bases 1230-1249 for N; bases 2141-2166 for P; bases 3002-3032 for M; bases 4680-4710 for F; bases

15   5440-5467 for M2; bases 6077-6107 for SH; bases 6912-6942 for G; and bases 13223-13253 for L). A consensus sequence is shown underneath (SEQ ID NO: 28). For positions where alternative assignments can occur, these are listed below the consensus (SEQ ID NOs: 29 and 30). By this analysis, the major element of the GE signal is contained within 12-13 nucleotides, of which positions 1-3 and 10-12 are exactly conserved.

20   FIG. 9 is a diagram illustrating the construction of a transcription cassette containing the ORF for jellyfish GFP under the control of HMPV transcription GS and GE signals, and insertion of this cassette into the HMPV strain 83 antigenomic cDNA clone following antigenomic position 41. Sequence representing part of the 3´ leader region (bases 23-41 of SEQ ID NO: 1) and the putative N GS signal and beginning of the putative N ORF (bases 42-66 of SEQ ID NO: 1) is shown at the

25   bottom. The GFP transcription cassette consists of a 714-nucleotide cDNA containing the GFP ORF flanked on the upstream side by 16 nucleotides (which include the initiating ATG of the GFP ORF) representing the conserved putative N GS signal (bases 42-57 of SEQ ID NO: 1) and flanked on the downstream side by 13 nucleotides representing the putative F GE signal (bases 4685-4697 of SEQ ID NO: 1; which include the termination codon of the GFP ORF) followed by the trinucleotide AGT

30   designed to function as an intergenic (IG) region. The total length of the transcription cassette including the trinucleotide intergenic region is 748 nucleotides. The F GE signal contains a naturally occurring PacI site (italicized).

       FIG. 10 is a diagram illustrating the construction of expression vectors comprising support plasmids that contain ORFs corresponding to the proposed nucleocapsid and polymerase proteins of

35   HMPV strain 83: N, P, M2-1 and L. These ORFs were clarified in part based on sequence relatedness to the respective RSV ORF (see, for example, Figure 5). Each ORF was placed under the control of a

T7 transcription promoter (T7p) and terminator (T7t). The L cDNA was assembled from two overlapping pieces. Each ORF conformed to the consensus sequence of HMPV 83.

FIG. 11 is a diagram showing the strategy for recovery of a recombinant version of HMPV strain 83, in this case expressing GFP as an added marker gene. Plasmids encoding the HMPV-GFP

5   antigenome and the N, P, L and M2-1 support plasmids are transfected into BSR T7/5 cells, which are a derivative of the baby hamster kidney-21 (BHK-21) cell line that was engineered to constitutively express T7 RNA polymerase (Buchholz et al., J. Virol. 73:251-259. 1999). The support plasmids were constructed to encode the proposed HMPV nucleocapsid and polymerase proteins. By this strategy, the expression of the antigenome together with HMPV nucleocapsid/polymerase

10   proteins provides the components for the self-assembly of HMPV nucleocapsids functional for transcription and RNA replication. These nucleocapsids then express all of the HMPV RNAs and proteins, leading to a productive infection that produces rHMPV.

FIG. 12A is a digitized image of an agarose gel showing that the recovered recombinant HMPV-GFP (strain 83) contains the NheI site that was engineered into the antigenome cDNA and is

15   not present in biologically-derived strain 83 parent. LLC-MK2 cells in T25 flask were infected with an aliquot of the supernatant from the transfected BSR T7/5 cells (first passage) and incubated at 32°C in the presence of 5 µg/ml trypsin. Eleven days post-infection, 1 ml of the supernatant was harvested, clarified by centrifugation at 1500 rpm for 5 minutes and used to extract the viral genomic RNA with the QIAamp viral RNA purification kit (QIAGEN) according to the manufacturer's

20   recommendations. The RNA was subjected to reverse transcription (RT) using a positive sense primer designed to hybridize within the M gene (nucleotides 2719 to 2738 in the HMPV antigenome exclusive of GFP). Polymerase chain reaction (PCR) was performed using the RT primer and a reverse primer designed to hybridize within the F gene (nucleotides 3894 to 3876 in the HMPV antigenome exclusive of GFP). An aliquot of each RT-PCR reaction was subjected to digestion with

25   NheI. The RT-PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. Lanes: 1 and 7, molecular weight marker; 2, a negative control in which RNA from the recombinant HMPV-GFP-infected cell supernatant was assayed with the omission of RT enzyme; lanes 3 and 4, RT-PCR products from RNA representing recombinant HMPV-GFP and biologically-derived HMPV, respectively; lanes 5 and 6, RT-PCR products from RNA representing recombinant

30   HMPV-GFP and biologically-derived HMPV, respectively, that had been subjected to digestion with NheI.

FIG. 12B is a digitized image of a nylon membrane illustrating Northern blot analysis of GFP mRNA expressed by the rHMPV-GFP virus. LLC-MK2 cells were mock-infected (lanes 1 and 5) or infected at an MOI of 3 PFU per cell with biologically-derived HMPV83 (lanes 2 and 6),

35   rHMPV (lanes 3 and 7), or rHMPV-GFP (lanes 4 and 8). Three days later, total intracellular RNA was isolated, electrophoresed on 1% agarose-formaldehyde gels, transferred to charged nylon and analyzed by hybridization to double-stranded ^{32}P-labeled DNA probe specific to the GFP (lanes 1-4)

or M (lanes 5-8) gene. The identities and calculated sizes of individual RNA species are indicated. This showed that the GFP transcription cassette was expressed predominantly as a single mRNA of the appropriate size to be a monocistronic GFP mRNA.

FIG. 13 is a graph illustrating multi-step growth of the recovered recombinant HMPV

5 (strain 83) (rHMPV) compared with biologically-derived HMPV 83. LLC-MK2 cells were inoculated with 0.01 plaque forming units (pfu) per cell and incubated at 32°C in the presence of 5 μg/ml trypsin. Aliquots were taken at 24 h intervals, flash-frozen, and viral titers were measured by plaque assay and immunostaining with convalescent serum from HMPV-infected hamsters. Each time point was represented by two wells, and each virus titration was done in duplicate. Means are shown.

10 FIG. 14 is a graph illustrating multi-step growth of recombinant HMPV-GFP compared to recombinant HMPV in LLC-MK2 cells, performed as described above for Figure 13. Two separate infections (a and b) were monitored for each virus, and each aliquot was titrated in duplicate and the mean is shown.

FIG. 15 is a diagram illustrating deletion of the SH and G genes singly and in combination

15 from rHMPV and rHMPV-GFP (strain 83), each containing introduced *BsiW*1 and *BsrG*1 restriction sites. The region of the antigenomic cDNA clone containing the putative SH and G ORFs is illustrated, together with proposed GS and GE transcription signals and proposed intergenic regions. *BsiW*1 and *BsrG*1 sites were introduced into the putative M2-SH and SH-G, respectively, intergenic regions of HMPV-GFP. In each case, the mutant sequence (nucleotide substitutions in small case) is

20 shown below the wild type sequence. The restriction sites are numbered according to the position of the first residue in the wild type HMPV sequence (SEQ ID NO: 1) exclusive of GFP, a convention that will be followed throughout this disclosure. The naturally-occurring *Acc*651 sequence also is shown. Note that cleavage of each of these restriction sites by its cognate enzyme leaves a compatible overhang, namely GTAC, that is compatible with each of the others. Thus, the genes can be readily

25 deleted by cutting with the appropriate pair of enzymes and religating. This results in small changes in the length of the relevant intergenic region, as noted. Each of these antigenomic cDNAs was successfully used to recover infectious mutant virus.

FIG. 16 is a digitized image of an agarose gel showing that the recovered recombinant HMPV-GFP ΔSH, ΔG and ΔSH/G viruses each contain a deletion of the appropriate size in the

30 region of the SH and G genes. Following the procedure described above for Figure 12, viral RNA was subjected to RT-PCR using primers that span the SH and G genes; specifically a positive sense primer designed to hybridize within the M2 and a negative sense primer designed to hybridize within the L gene. The RT-PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. Lanes: 1 and 8, molecular weight markers; lane 3, a negative control in which

35 RNA from recovered recombinant HMPV was subjected to the RT-PCR procedure with the omission of RT enzyme; lanes 2 and 4-7, RT-PCR products representing the indicated viruses.

FIG. 17A is a graph illustrating multi-step growth of rHMPV-GFP-ΔSH, rHMPV-GFP-ΔG,

and rHMPV-GFP-ΔSH/G compared to rHMPV and rHMPV-GFP, performed in LLC-MK2 cells as

described above for Figure 13. Since the deletions had been introduced into rHMPV-GFP, rHMPV-

GFP is the "wild type" equivalent for comparison.

5   FIG. 17B is a graph illustrating the efficiency of replication of biologically-derived

HMPV83 and recombinantly-derived rHMPV, rHMPVΔSH, rHMPVΔG, and rHMPVΔSH/G in the

upper (nasal turbinates) and lower (lungs) respiratory tract of Golden Syrian hamsters. Animals in

groups of 12 were infected intranasally with the following viruses at a dose of 5.0×10^5 $TCID_{50}$ per

animal in 0.1 ml: biologically derived HMPV83, rHMPV, rHMPVΔSH, and rHMPVΔSH/G, and the

10   following virus at 1.6×10^5 $TCID_{50}$ per animal in 0.1 ml: rHMPVΔG. Six animals from each group

were sacrificed on days 3 and 5 and the nasal turbinates and lungs were recovered and analyzed by

limiting dilution to determine the viral titer. This showed that the rHMPV replicated in vivo with an

efficiency similar to that of its biologically-derived parent (HMPV83), that the replication of the

rHMPVΔSH virus was not significantly reduced, and that the replication of the rHMPVΔG and

15   ΔSH/G viruses were strongly reduced, although virus replication was detectable in each case.

FIG. 17C is a graph illustrating the protective efficacy of immunization with the indicated

HMPV deletion mutant viruses. Golden Syrian hamsters in groups of 6 were infected as described in

Figure 17B and, 27 days later, serum samples were taken and analyzed to determine the titers of

HMPV-neutralizing serum antibodies. These titers are shown at the bottom. The animals were then

20   challenged on day 28 by the intranasal instillation of 5.0 log_{10} $TCID_{50}$ per animal of biologically-

derived wild type HMPV83. Three days later, the animals were sacrificed and the nasal turbinates

and lungs were harvested, homogenized, and analyzed by limiting dilution to determine virus titers.

This showed that there was no detectable replication of the challenge virus in animals that has

received rHMPV or rHMPVΔSH, whereas animals that initially had received rHMPVΔG or

25   rHMPVΔSH/G had no detectable challenge virus replication in the lungs and had reduced replication

in the nasal turbinates compared to the control that had not been previously infected.

FIG. 18A is a diagram illustrating ablation of the putative M2-2 ORF in infectious rHMPV

and rHMPV-GFP (strain 83), each containing an introduced BsiWI site. The region of the

antigenomic cDNA clone containing the putative M2-1 and M2-2 ORF is illustrated, with each ORF

30   depicted by a solid horizontal line. Two nucleotide substitutions were made at positions 5236 and

5248 (SEQ ID NO: 1; numbered according to the complete antigenomic cDNA exclusive of GFP)

that ablate two potential translation initiation codons for the M2-2 ORF. A third substitution was

made at position 5272 (SEQ ID NO: 1) that introduces an in-frame stop codon. In addition,

nucleotides 5289-5440 (SEQ ID NO: 1) were deleted, removing most of the M2-2 ORF. The ΔM2-2

35   mutant was successfully recovered in both the rHMPV and rHMPV-GFP backbones and designated

rHMPVΔM2-2 and rHMPV-GFPΔM2-2, respectively.

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FIG. 18B is a graph illustrating evaluation of multi-step growth kinetics of the recovered viruses *in vitro*. The rHMPVΔM2-2 and rHMPV-GFPΔM2-2 viruses were compared with rHMPV and rHMPV-GFP with regard to multi-step growth kinetics and yield in LLC-MK2 cells as described above for Figure 13. rHMPVΔM2 clones #1 and #2 represent virus derived independently from two

5   sister cDNA clones.

FIG. 18C is a digitized image of a nylon membrane illustrating Northern blot analysis of intracellular RNAs expressed by rHMPV-GFP and rHMPV-GFPΔM2-2. Replicate monolayers of LLC-MK2 cells were infected at an MOI of 3 PFU per cell with either virus as indicated and incubated at 32°C. Monolayers were harvested at 9, 24, 36, 48, and 72 h post infection as indicated

10   and processed for purification of intracellular RNA. The RNA preparations were analyzed by Northern blot hybridization with strand-specific riboprobes representing the N gene: the upper panels detect hybridization with the negative-sense probe and thus represent antigenome and N-related mRNA, as indicated, and the bottom panels detect hybridization of a replicate set of gel lanes (that had been transferred in parallel onto the same membrane, which was then cut and the replicate sets

15   hybridized separately) with positive-sense riboprobe and represent genome. Beneath the Northern blot are calculations from four experiments involving hybridization with a probe specific for N or for F, as indicated. In each experiment, the amount of total N-related or F-related mRNA for each time point was divided by the amount of antigenome from a replicate gel lane (that had been transferred in parallel onto the same membrane, which was then cut and the replicas hybridized separately). Then,

20   each time point for the rHMPV-GFPΔM2-2 virus was normalized relative to the corresponding time point for rHMPV-GFP as 1.0. Thus, this provides a comparison of the efficiency of mRNA expression for the ΔM2-2 mutant compared to wild type HMPV.

FIG. 19 is a diagram illustrating the introduction of single amino acid substitutions into the Cys3-His1 motif of the M2-1 protein of rHMPV-GFP (strain 83). This motif consists of three

25   cysteine residues (C7, C15 and C21) and one histidine residue (H25); it is also found in RSV and other pneumoviruses, and thus represents a conserved pneumovirus motif. The amino acid sequence shown is from methionine (M) 1 to asparagine (N) 26 of the deduced complete M2-1 amino acid sequence (SEQ ID NO: 4). Each of these mutants was successfully recovered as infectious recombinant virus.

30   FIG. 20 is a graph illustrating multi-step growth of derivatives of rHMPV-GFP containing mutations in the Cys3-His1 motif of the M2-1 ORF, compared to rHMPV-GFP and rHMPV, performed in LLC-MK2 cells as described above for Figure 13. The mutants involve single amino acid point mutations in the Cys3-His1 motif: C7S, Y9S, C15S, N16S and H25S. The mutations were introduced into rHMPV-GFP, and thus rHMPV-GFP is the "wild type" or parental equivalent for

35   comparison.

FIG. 21 is a diagram illustrating silencing of the M2-1 ORF in rHMPV-GFP (strain 83) by replacing the ATG translational start site (shaded) with a TAG translational termination codon, and

by replacing additional ATG triplets (underlined) in each reading frame with stop codons. The top line shows the nucleotide sequence of the upstream end of the M2-1 mRNA (corresponding to nucleotides 4711 to 4775 in the complete HMPV antigenome sequence; SEQ ID NO: 1). The second line shows nucleotide substitutions (lower case letters) introduced into the M2-1 sequence of

5   rHMPV-GFP to yield rHMPV-GFP-ΔM2-1. The third line shows the first 17 amino acids of the HMPV M2-1 protein (SEQ ID NO: 4), and the fourth line shows coding changes introduced by the point mutations in rHMPV-GFP-ΔM2-1, with termination codons in the M2-1 ORF indicated by asterisks. Note that the next in-frame ATG in the M2-1 ORF is at codon 134 out of the total of 187 codons in the ORF (SEQ ID NO: 4). This mutant was successfully recovered as infectious

10   recombinant virus.

FIG. 22A is a diagram illustrating deletion of the complete M2 gene in infectious rHMPV-GFP (strain 83) containing an introduced *BsiW*I site. The region of the antigenomic cDNA clone containing the putative M2-1 and M2-2 ORF is illustrated, with each ORF depicted by a solid horizontal line. Nucleotides 4701 to 5459 (SEQ ID NO: 1), representing a total of 759 nucleotides,

15   were deleted, resulting in the mutant rHMPV-GFP-ΔM2(1+2). This mutant was successfully recovered as infectious recombinant virus.

FIG. 22B is a graph illustrating a comparison of the efficiency of growth of viruses with alterations in the coding of M2-1 and/or M2-2 proteins in LLC-MK2 cells (upper panel), which are competent for expressing type I interferons versus Vero cells (lower panel), which lack the interferon

20   structural genes. Cells were infected with 0.01 PFU per cell and incubated at 32°C in the presence of 5μg/ml trypsin. Aliquots were taken at 24 h intervals, flash-frozen, and viral titers were measured by plaque assay and immunostaining with convalescent serum from hamsters that had been infected with HMPV.

FIG. 22C is a chart demonstrating increased sensitivity of HMPV ΔM2(1+2) and ΔM2-2

25   mutants to type I interferon. Replicate cultures of Vero cells were treated overnight with the indicated amount of interferon per 1.5 X 10⁶ cells. The cells were infected with the indicated amount of wild type rHMPV-GFP, rHMPV-GFPΔM2(1+2), rHMPV-GFPΔM2-2, or rgRSV, the last being a recombinant RSV that expresses the GFP gene. The GFP marker was used to visually monitor virus growth during the experiment to judge the appropriate time of harvest, and is otherwise irrelevant to

30   this experiment. The cells were harvested on day 4 and the yield of each virus was determined and compared with additional replicate cultures that had been mock-interferon treated, infected and harvested in parallel. The results are expressed as the fold reduction of each interferon-treated culture compared to its untreated counterpart.

FIG. 23A is a diagram illustrating the construction of two rearrangements of the genes of

35   rHMPV-GFP (strain 83). The wild type gene order is shown on top for the M-L region of the rHMPV-GFP antigenomic cDNA clone. The restriction sites shown are the ones introduced as illustrated above in Figures 7 and 15 (the restriction sites are numbered according to the position of

the first residue in the wild type HMPV sequence, SEQ ID NO: 1, exclusive of GFP), and were used
to rearrange the order of the putative SH and G gene pair. Religations involving *Nhe*I were not
compatible with the other sites and necessitated fill-in of each end followed by blunt end ligation.
Two examples of gene rearrangements are shown: in the mutant called "Order 1", the positions of the

5   SH-G gene pair and the F-M2 gene pair were swapped, resulting in a local gene order that mimics
that of RSV, namely M-SH-G-F-M2-L. This mutant was successfully recovered as infectious virus.
In a subsequent mutant, "Order 2", the same RSV-like gene order was achieved, but the SH-G gene
pair was duplicated.

FIG. 23B is a graph illustrating the multi-step growth kinetics of one of these mutants,

10   rHMPV-GFP-Order #1 compared to its direct parent rHMPV-GFP, as well as rHMPV, performed as
described above for Figure 13. Clones #3 and #13 represent viruses derived independently from two
sister cDNA clones.

FIG. 24A is a diagram illustrating shifting of the F and/or G genes of HMPV (strain 83)
from their natural positions as the fourth and seventh genes, respectively, to promoter-proximal

15   positions 1 or 2. As illustrated in the upper box, the putative F and G ORFs were engineered to be
flanked by putative GS (bases 3054-3069 and 6219-6234 of SEQ ID NO: 1, respectively) and GE
(bases 4685-4697 of SEQ ID NO: 1) signals and inserted individually into the HMPV antigenomic
cDNA clone. Sequence representing part of the 3′ leader region (bases 23-41 of SEQ ID NO: 1) and
the putative N GS signal and beginning of the putative N ORF (bases 42-66 of SEQ ID NO: 1) is

20   shown. Note that the choice of transcription signals and insertion site was the same as for the
rHMPV-GFP construct shown above in Figure 9, and places the inserted gene as the first in the gene
order. As illustrated in the lower box, the putative F and G ORFs were inserted as a pair, in the order
G1-F2 or its converse F1-G2, using the same transcription signals and insertion site as in the single-
gene rearrangements. Infectious virus was successfully recovered from each of the constructs. These

25   viruses were designated rHMPV-F1, -G1, -F1G2, and G1F2, with the number indicating the position
of the gene in the gene order. In the virus designation, the "-" symbol preceding the shifted gene
indicates that the shifted gene was removed from its normal position in the gene order.

FIG. 24B is a graph illustrating multi-cycle growth kinetics of the recovered viruses *in vitro*.
The recovered rHMPV-G1, F1, F1G2, and G1F2 viruses were compared to their rHMPV parent with

30   regard to multi-step growth in LLC-MK2 cells as described above for Figure 13.

FIG. 25A is a diagram showing representations of rHMPV in which one or more extra
copies of the F and/or G gene was placed in promoter proximal positions 1, 2 or 3 in addition to the F
and/or G gene present in the normal genome position. The G or F genes were inserted individually
into promoter proximal position 1 (rHMPV+G1 or +F1), or were inserted as a pair into positions 1

35   and 2 in each of the two possible orders (rHMPV+G1F2 or +F1G2), or were inserted as the pair
G1F2 with an additional copy of F in the third position (rHMPV+G1F2,3). The detailed structure of
the gene insertions were as shown in Figure 24A. In the designations, the "+" symbol before the

named gene or genes indicates that it/they were additional to the one or ones in the normal position. The added genes are shaded.

FIG. 25B is a graph illustrating multi-cycle growth kinetics *in vitro*. The recovered viruses were compared to their rHMPV parent and to rHMPV-GFP with regard to multi-step growth in LLC-MK2 cells as described above for Figure 13.

FIG. 25C is a digitized image of a nylon membrane illustrating Northern blot analysis of intracellular mRNAs expressed by rHMPV+G1F23. LLC-MK2 cells were mock-infected (lanes 1, 5 and 9) or infected at an MOI of 3 PFU per cell with HMPV83 (lanes 2, 6 and 10), rHMPV (lanes 3, 7 and 11), or rHMPV+G1F23 (lanes 4, 8 and 12). Three days later, total intracellular RNA was isolated, electrophoresed on 1% agarose-formaldehyde gels, transferred to charged nylon and analyzed by hybridization to double-stranded ^{32}P-labeled DNA probe specific to the F (lanes 1-4), G (lanes 5-8), or M (lanes 9-12) gene. The identities and calculated sizes of individual RNA species are indicated.

FIGS. 26A-26F are diagrams illustrating amino acid locations in the strain 83 HMPV L protein (SEQ ID NO: 7) that are targets for mutagenesis based on mapping of attenuating mutations in a heterologous virus (HRSVA2, SEQ ID NO: 13; HPIV3, SEQ ID NO: 14; HPIV1, SEQ ID NO: 15; or BPIV3, SEQ ID NO: 16, as indicated). HMPV strain 001, SEQ ID NO: 12, is included in the alignments for comparison. Numbers at the left indicate the positional reference of the first residue in the partial amino acid sequence shown, and numbers to the right indicate the positional reference number of the terminal residue in the partial sequence. The more highly conserved or similar residues are denoted by shading. Dashes indicate a gap in the particular sequence introduce to maximize the alignment.

FIG. 27 is a graph illustrating multi-step growth of rHMPV-GFP containing the F456L mutation (see Figure 26A), compared to rHMPV, performed in LLC-MK2 cells as described above for Figure 13. Clones #3 and #7 represent viruses derived independently from two sister cDNA clones.

FIG. 28 is a diagram illustrating the GS signal of the M2 gene of wild type (WT) RSV (SEQ ID NO: 31), and a highly-attenuating T to C nucleotide substitution at position nine in the GS signal of the M2 gene of the attenuated cpts248/404 RSV mutant (Whitehead *et al.*, J. Virol. 72:4467-4471, 1998). There is a corresponding T residue that is highly conserved among all of the putative GS signals of HMPV. The individual GS motifs are named according to the putative ORF that each precedes (SEQ ID NO: 1, bases 41-57 for N; bases 1249-1265 for P; bases 2166-2182 for M; bases 3053-3069 for F; bases 4710-4726 for M2; bases 5467-5483 for SH; bases 6218-6234 for G; and bases 7119-7135 for L). The sequence at the bottom (SEQ ID NO: 32) indicates in upper case letters those positions in the putative HMPV GS signal that are exactly conserved among all of the signal in strain 83, which includes the T at position nine.

FIG. 29 is a diagrammatic representation of the genome structures of biologically-derived
HMPV strains 83 and CAN98-75 (hereafter referred to as 75 or CAN75), representing the two
proposed genetic HMPV subgroups and thus illustrating the diversity that must be considered in
designing an HMPV immunogenic composition. Individual genes are indicated by boxes, with gene

5   lengths and boundaries within the complete genomic sequence given in nucleotides together with the
unmodified amino acid length (italics) of the deduced protein (in the case of M2, two predicted
proteins, M2-1 and M2-2). The nucleotides lengths of the extragenic 3′ leader, 5′ trailer and
intergenic regions are underlined.

FIG. 30 is a diagram illustrating the construction of chimeric viruses constructed with genes

10   of HMPV and APV, which represent potential vaccine candidates. Part A illustrates the genome of
HMPV83 and derivatives in which the N, P or M ORF is replaced individually by its APV
counterpart (unshaded boxes). Parts B, C and D illustrate the structures of cDNAs containing the N, P
and M genes, respectively of HMPV (upper line in each box) compared to a version containing the
indicated substituted APV ORF (lower line in each box). Each ORF is shown as a shaded box and is

15   flanked by HMPV GS (SEQ ID NO: 1, bases 12-57 for the leader/N GS signal; bases 1235-1265 for
the N GE/P GS signal; and bases 2146-2182 for the P GE/M GS signal) and GE (SEQ ID NO: 1,
bases 1235-1242 for the partial N GE signal; bases 2146-2151 for the partial P GE signal; and bases
3007-3037 for the M GE signal) transcription signals (boxed). In the case of the APV M gene, 64 nt
of noncoding sequence that separates the ORF from the GE signal have been deleted (-64 nts nc). The

20   GE and GS signals in turn are flanked by restriction sites selected from the following list, as
indicated: BbsI, BsmBI, BfuAI, BbsI. Each of these sites consists of a recognition sequence
(underlined) and has the property of cutting in a sequence-independent fashion at a second site
outside of the recognition site (underlined tetranucleotide, bold) to leave a 4-nt 5′-protruding
overhang. Here, the overhangs were TAAT or GTAG, as indicated, which matches the natural HMPV

25   assignments at these positions. Part E illustrates a novel assembly strategy that utilizes the 4-nt
overhangs, plus the flanking MluI and NheI sites, for ligation of the N, P and M cDNAs
simultaneously into the HMPV backbone. By this strategy, this can be used to mix HMPV and APV
N, P and M genes in whatever combinations desired to make replacements of single HMPV genes (as
in part A) or to replace two or three genes simultaneously.

30   FIG. 31 is a diagram showing the comparison of the predicted gene boundaries, cis-acting
GS and GE signals, and intergenic regions of HMPV strains 83 (SEQ ID NO: 1) and 75 (SEQ ID
NO: 2), shown as positive-sense sequence. The top alignment shows the leader/N gene boundary,
specifically the last 16-18 nucleotides of the leader region followed by the first 15 nucleotides of the
N gene. The second alignment shows the N/P gene boundary, specifically the last 15 nucleotides of

35   the N gene, followed by the adjoining intergenic region, followed by the first 15 nucleotides of the P
gene. Subsequent alignments show the P/M, M/F, F/M2, M2/SH, SH/G, G/L, and L/trailer
boundaries. Conserved sequence motifs at the end (gene end) and beginning (gene start) of each gene

are indicated in bold upper case, and a consensus is given below (SEQ ID NOs: 34 and 35).
Positions within these conserved motifs are numbered. Translational stop and start codons are
underlined. Intergenic sequences are shown: in the case of the longer intergenic regions, only the
first 5 nucleotides on the upstream and downstream ends are shown, and the number of nucleotides
5   not shown is indicated. For each assignment in the consensus sequence, no more than two of the
sequence pairs (HMPV 83 versus 75) could have a heterologous assignment in both subgroup
sequences.

       FIG. 32 is a chart illustrating the percent amino acid and nucleotide sequence identity (the
latter given in parentheses) between the predicted proteins and ORFs of: HMPV 83 versus HMPV 75,
10 representing a comparison across subgroups; HMPV83 versus HMPV 00-1, representing a
comparison within a subgroup; and RSV A2 versus RSV B1, representing a comparison across the
two well-characterized RSV antigenic subgroups A and B, which serve here as a benchmark for
assessing the magnitude and anticipated significance of the differences in the HMPV subgroups.
Amino acid sequence identities were calculated based on the complete predicted proteins; in the case
15 of G and SH, overhangs on the carboxy-terminal side of alignments due to length differences were
not included in the calculations. Nucleotide sequence identities for the corresponding ORFs are
shown in parentheses and are based on the protein-coding sequence exclusive of flanking noncoding
sequence. For comparison, the HMPV intergenic regions were 48% identical between subgroups and
noncoding gene sequences exclusive of conserved transcription signals were 54% identical, compared
20 to values of 42% and approximately 50% for RSV (Johnson et al., J. Gen. Virol. 69:2901-2906,
1988).

       FIGS. 33A-33B are diagrams showing the alignment of the amino acid sequences of the SH
(Figure 33A) and G (Figure 33B) proteins of HMPV strains 75 (SEQ ID NOs: 8 and 9, respectively),
83 (SEQ ID NOs: 5 and 6, respectively) and 00-1 (SEQ ID NOs: 10 and 11, respectively). This
25 includes comparison between (75 versus 83) and within (83 versus 00-1) the proposed HMPV genetic
subgroups. For strains 75 and 00-1, assignments that differ from that of 83 are shown; dashes
indicate gaps introduced to maximize the alignment or to denote the absence of corresponding amino
acids. Stars underneath each alignment denote amino acid identity among all three sequences; small
dots indicate amino acid similarity among all three. Proposed signal/transmembrane domains are
30 boxed. Motifs for N-linked carbohydrate are underlined (N-X-T/S, where X is not proline). In panel
A, cysteine residues conserved among all three SH proteins are indicated with large dots, and
potential sites for O-linked glycosylation (Hansen et al., Glycoconj. J. 15:115-130, 1998) of the SH
proteins are as follows: HMPV 83, 75, 77, 78, 81; HMPV 75, 78, 79, 81; 00-1, 77, 78, 81. The
ectodomain of the G protein of each virus contained more than 40 potential acceptor sites for O-
35 linked carbohydrate.

       FIG. 34 is a diagram illustrating the design of a chimeric HMPV virus bearing antigens
specific to HMPV strains that represent two different antigenic subgroups of HMPV. The strain 83

antigenomic cDNA clone was used as the backbone (sequence representing part of the 3' leader

region (bases 23-41 of SEQ ID NO: 1) and the putative N GS signal and beginning of the putative N

ORF (bases 42-66 of SEQ ID NO: 1) is shown), and a transcription cassette was designed that

consists of the ORF encoding the putative G, F, or SH surface proteins of strain 75 under the control

5   of transcription signals from strain 83 (which including bases 42-57 of SEQ ID NO: 1, representing

the conserved putative N GS signal, and bases 4685-4697 of SEQ ID NO: 1, representing the putative

F GE signal). This transcription cassette is then inserted into the strain 83 backbone at a promoter-

proximal site. This strategy for constructing a bivalent immunogenic construct employs the same

strain 83 transcription signals and insertion site as shown for the HMPV-GFP construct in Figure 9.

10   FIG. 35 is a graph illustrating the daily level of replication of HMPV strains 75 and 83 in

the upper and lower respiratory tract of African green monkeys. Mean daily virus titers in the

nasopharyngeal (upper panel) or tracheal lavage (lower panel) specimens obtained on the indicated

day post-inoculation from animals infected simultaneously by the intranasal and intratracheal routes

with 105 TCID50 of the indicated virus: strain 75 (●), strain 83 (■).

15   FIGS. 36A-36B are a diagram and digitized images showing the construction and

expression of a recombinant HPIV1 vector expressing the F protein of HMPV CAN83 (rHPIV1-F₈₃).

The HPIV1 genome was modified by the creation of an MluI restriction site (HPIV1 nt 113-118) one

nucleotide prior to the translational start codon of the N ORF (HPIV1 nt 119-121). The F ORF of

HMPV strain CAN83 (1620 nt in length and encoding a 539 aa polypeptide; bases 3054-4697 of SEQ

20   ID NO: 1) was engineered by PCR to be followed by the tetranucleotide TAAT and an HPIV1 gene

junction consisting of a GE signal, CTT intergenic region, and a GS signal. The length of the entire

cassette was 1656 nt and was designed, upon insertion into the MluI site, to conform to the rule of six

and to maintain the HPIV1 GS signal sequence phasing. GS and GE signals are indicated (FIG.

36A). A portion of the predicted viral promoter that lies within the N gene is shown (FIG. 36A).

25   LLC-MK2 cells were infected with rHPIV-F83, HMPV CAN83, rHPIV1 or mock-infected, as

indicated, incubated for 72 hr, and analyzed by indirect immunofluorescence using anti-HMPV

polyclonal hamster serum or a mixture of two mouse monoclonal antibodies to the HPIV1 HN

protein, as indicated (Fig. 36B).

FIGS. 37A-37D are the complete sequence of the genome of biologically-derived strain 83,

30   shown as positive sense DNA (SEQ ID NO: 1). See also, GenBank Accession No. AY297749.

FIGS. 38A-38D are the complete sequence of the genome of recombinant strain 83 bearing

a transcription cassette expressing GFP, which recombinant strain exemplifies rHMPV-GFP of the

current disclosure (SEQ ID NO: 3).

FIGS. 39A-39D are the complete sequence of the genome of biologically-derived,

35   biologically-cloned HMPV CAN75 (SEQ ID NO: 2). See also, GenBank Accession No. AY297748.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. In the accompanying sequence listing:

SEQ ID NO: 1 shows the nucleic acid sequence of the genome of biologically-derived HMPV (strain 83) as positive sense DNA.

SEQ ID NO: 2 shows the nucleic acid sequence of the genome of biologically-derived HMPV (strain 75) as positive sense DNA.

SEQ ID NO: 3 shows the nucleic acid sequence of the genome of recombinant HMPV (strain 83) with a transcription cassette expressing GFP as positive sense DNA.

SEQ ID NO: 4 shows the amino acid sequence of the HMPV (strain 83) M2-1 protein.

SEQ ID NO: 5 shows the amino acid sequence of the HMPV (strain 83) SH protein.

SEQ ID NO: 6 shows the amino acid sequence of the HMPV (strain 83) G protein.

SEQ ID NO: 7 shows the amino acid sequence of the HMPV (strain 83) L protein.

SEQ ID NO: 8 shows the amino acid sequence of the HMPV (strain 75) SH protein.

SEQ ID NO: 9 shows the amino acid sequence of the HMPV (strain 75) G protein.

SEQ ID NO: 10 shows the amino acid sequence of the HMPV (strain 00-1) SH protein.

SEQ ID NO: 11 shows the amino acid sequence of the HMPV (strain 00-1) G protein.

SEQ ID NO: 12 shows the amino acid sequence of the HMPV (strain 00-1) L protein.

SEQ ID NO: 13 shows the amino acid sequence of the HRSVA2 L protein.

SEQ ID NO: 14 shows the amino acid sequence of the HPIV3 L protein.

SEQ ID NO: 15 shows the amino acid sequence of the HPIV1 L protein.

SEQ ID NO: 16 shows the amino acid sequence of the BPIV1 L protein.

SEQ ID NO: 17 shows the nucleic acid sequence of the HMPV (strain 00-1) 3' leader.

SEQ ID NO: 18 shows the nucleic acid sequence of the HMPV (strain 83) 3' leader.

SEQ ID NO: 19 shows the nucleic acid sequence of the APV 3' leader.

SEQ ID NO: 20 shows the nucleic acid sequence of the RSV 3' leader.

SEQ ID NO: 21 shows the nucleic acid sequence of the HMPV (strain 00-1) 5' trailer.

SEQ ID NO: 22 shows the nucleic acid sequence of the HMPV (strain 83) 5' trailer.

SEQ ID NO: 23 shows the nucleic acid sequence of the APV 5' trailer.

SEQ ID NO: 24 shows the nucleic acid sequence of the RSV 5' trailer.

SEQ ID NO: 25 shows the nucleic acid sequence of the HMPV (strain 83) GS consensus sequence.

SEQ ID NO: 26 shows the nucleic acid sequence of the HMPV (strain 83) GS consensus sequence, with alternative nucleic acid assignments.

SEQ ID NO: 27 shows the nucleic acid sequence of the HMPV (strain 83) GS consensus sequence, with alternative nucleic acid assignments.

5   SEQ ID NO: 28 shows the nucleic acid sequence of the HMPV (strain 83) GE consensus sequence.

SEQ ID NO: 29 shows the nucleic acid sequence of the HMPV (strain 83) GE consensus sequence, with alternative nucleic acid assignments.

SEQ ID NO: 30 shows the nucleic acid sequence of the HMPV (strain 83) GE consensus
10   sequence, with alternative nucleic acid assignments.

SEQ ID NO: 31 shows the nucleic acid sequence of the RSV M2 GS sequence.

SEQ ID NO: 32 shows the nucleic acid sequence of the HMPV (strain 83) GS consensus sequence, with uniformly conserved bases.

SEQ ID NO: 33 shows the nucleic acid sequence of a polylinker used in the cloning and
15   expression of rHMPV.

SEQ ID NO: 34 shows the nucleic acid sequence of the HMPV (strain 83/75) GE consensus sequence.

SEQ ID NO: 35 shows the nucleic acid sequence of the HMPV (strain 83/75) GS consensus sequence.

20   SEQ ID NO: 36 shows the nucleic acid sequence of the genome of HMPV (strain 00-1) as positive sense DNA.

DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS

25   In one embodiment, methods and compositions are provided for producing an infectious, self-replicating, recombinant HMPV from one or more isolated polynucleotide molecules encoding viral sequences. The methods and compositions generally involve coexpressing in a cell or cell-free system one or more expression vectors comprising a polynucleotide molecule that encodes a partial or complete, recombinant HMPV genome or antigenome and one or more polynucleotide molecules
30   encoding HMPV N, P, and L proteins, so as to produce an infectious HMPV particle. In certain embodiments, the methods and compositions for producing the recombinant HMPV further include expression of the M2-1 gene.

Typically, the polynucleotide molecule that encodes the recombinant HMPV genome or antigenome is a cDNA. Thus, disclosed herein are polynucleotides such as cDNAs and their
35   equivalents that encode a recombinant HMPV. Expression vectors and constructs that incorporate a polynucleotide molecule encoding a recombinant HMPV genome or antigenome are also disclosed herein.

The HMPV genome or antigenome, and the N, P, M2-1 and L proteins can all be produced from a single expression vector. However, the genome or antigenome can be produced by a separate expression vector, and the N, P, M2-1 and L proteins can be produced by one, two, or more additional expression vectors. One or more of the N, P, M2-1 and L proteins can be supplied by

5   expression of a recombinant HMPV genome or antigenome. These proteins can also be supplied by coinfection with the same or different HMPV. Thus, in several embodiments, one or more of the N, P, M2-1 and L proteins are from a heterologous HMPV.

Infectious, recombinant, self-replicating viral particles are disclosed that are produced according to the foregoing methods. These particles include complete viruses as well as viruses that

10   lack one or more non-essential proteins or non-essential portions (for example, a cytoplasmic, transmembrane or extracellular domain) of a viral protein. Viruses of the current disclosure that lack one or more such non-essential components (for example, a gene or genome segment from the HMPV SH, G, M2-2 open reading frame (ORF) or comparable accessory ORFs, or a segment of one or more other ORFs, or an intergenic or other non-coding or non-essential genome component) are

15   referred to herein as incomplete viruses or "subviral particles." Exemplary subviral particles can lack any selected structural element, including a gene, gene segment, protein, protein functional domain, etc. that is present in a complete virus (that is, an assembled virion including a complete genome or antigenome, nucleocapsid and envelope). For example, a subviral particle of the current disclosure can include an infectious nucleocapsid containing a genome or antigenome, and the products of the

20   N, P, and L genes. Other subviral particles are produced by partial or complete deletions or substitutions of non-essential genes and/or their products among other non-essential structural elements.

Complete viruses and subviral particles produced by the methods disclosed herein are typically infectious and self-replicative through multiple rounds of replication in a mammalian host amenable to infection by HMPV. These hosts include various *in vitro* mammalian cell populations,

25   *in vivo* animal models widely known and accepted in the art as reasonably predictive of HMPV activity in humans (including, mice, hamsters, cotton rats, non-human primates including African green monkeys and chimpanzees), and humans, including seronegative and seropositive infants, children, juveniles, and adults. However, viruses and subviral particles also can be produced that are

30   highly defective for replication *in vivo*.

In one embodiment, the polynucleotide molecule encodes a sequence of a wild type HMPV (either the genome or the antigenome). The term "wild type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily

35   designed the "normal" or "wild-type" form of the gene. In another embodiment, the genome or antigenome can include one or more mutations from a biologically derived mutant HMPV, or any combination of recombinantly-introduced mutations; including one or more polynucleotide

insertions, deletions, substitutions, or rearrangements that is/are selected to yield desired phenotypic effects in the recombinant virus.[1]

Thus, the recombinant HMPV genome or antigenome can be engineered using the methods disclosed herein to incorporate a recombinantly-introduced restriction site marker, or a translationally

5   silent point mutation for handling or marking purposes. In other embodiments, the polynucleotide molecule encoding the recombinant HMPV genome or antigenome can incorporate one or more recombinantly-introduced attenuating mutations. In specific examples, the recombinant HMPV genome or antigenome incorporates one or more recombinantly-introduced, temperature sensitive (ts) or host range (hr) attenuating (att) mutations.

10   The recombinant HMPV genome or antigenome can incorporate one or more attenuating mutations identified in a biologically derived mutant HMPV strain, or in another mutant nonsegmented negative stranded RNA virus. For example, a mutation in a L, M, N, P, M2-1, M2-2, SH, F, or G protein, or in an extragenic sequence selected from a 3' leader or in a control signal such as the M2 GS sequence. Where the mutation is of one or more particular amino acid residues, the

15   recombinant HMPV genome or antigenome can incorporate multiple nucleotide changes in the codons specifying the mutation to stabilize the modification against reversion.

The recombinant HMPV genome or antigenome can include an additional nucleotide modification specifying a phenotypic change, such as an alteration in growth characteristics, attenuation, temperature-sensitivity, cold-adaptation, plaque size, host-range restriction, or a change

20   in immunogenicity. These additional modifications can alter one or more of the HMPV N, P, M, SH, G, F, M2-1, M2-2 and/or L genes and/or a 3' leader, 5' trailer, a cis-acting sequence such as a GS or GE sequence, and/or intergenic region within the HMPV genome or antigenome. For example, one or more HMPV genes can be deleted in whole or in part, or expression of the genes can be reduced or ablated by a frameshift mutation, by a mutation that alters a translation start site, by introduction of

25   one or more stop codons in an ORF of the gene, or by a mutation in a transcription signal. In several embodiments, the recombinant HMPV genome or antigenome is modified by a partial or complete deletion of the HMPV SH, G, or M2-2 gene or ORFs, or one or more nucleotide changes that reduces or ablates expression of one or more HMPV genes yet yields a viable, replication competent, infectious viral construct. In other embodiments, the recombinant HMPV genome or antigenome is

30   modified to encode a non-HMPV molecule such as a cytokine, a T-helper epitope, a restriction site marker, or a protein of a microbial pathogen capable of eliciting a protective immune response in a mammalian host.

A recombinant HMPV genome or antigenome can include a partial or complete HMPV "vector" genome or antigenome combined with one or more heterologous genes or genome segments

35   (nucleic acid sequences) encoding one or more antigenic determinants of one or more heterologous pathogens to form a chimeric HMPV genome or antigenome. The heterologous genes or genome segments encoding the antigenic determinants can be added as supernumerary genes or genome

segments adjacent to or within a noncoding region of the partial or complete HMPV vector genome
or antigenome, or can be substituted for one or more counterpart genes or genome segments in a
partial HMPV vector genome or antigenome. The heterologous genes or genome segments can
include one or more heterologous coding sequences and/or one or more heterologous regulatory

5   elements comprising an extragenic 3' leader or 5' trailer region, a GS signal, GE signal, editing
region, translational start site, intergenic region, or a 3' or 5' non-coding region.

        In additional embodiments, the heterologous pathogen is one or more heterologous
pneumoviruses (for example, a heterologous HMPV or RSV) and the heterologous genes or genome
segments encodes one or more HMPV or RSV N, P, M, SH, G, F, M2-1, M2-2 and/or L proteins or

10   fragments thereof. Thus, the antigenic determinants can be from a heterologous HMPV or RSV G,
SH and F glycoproteins, and antigenic domains, fragments and epitopes thereof, that is/are added to
or substituted within the partial or complete HMPV genome or antigenome. In several examples,
genes encoding G, SH and F glycoproteins of a heterologous HMPV or RSV are substituted for
counterpart HMPV G, SH and F genes in a partial HMPV vector genome or antigenome. In

15   additional examples, genes encoding the G, SH, and F glycoproteins of a heterologous HMPV or
RSV are expressed in addition to the HMPV sequences. In this manner, a plurality of heterologous
genes or genome segments encoding antigenic determinants of multiple heterologous pneumoviruses
can be added to or incorporated within the partial or complete HMPV vector genome or antigenome.
In other embodiments, one or more genes from APV or a related pneumovirus exhibiting a host range

20   restriction in humans is used to replace the corresponding genes in humans to achieve an attenuated
derivative and other improved properties.

        The disclosed recombinant human metapneumoviruses (HMPVs) can be used to generate a
desired immune response against one or more HMPVs, or against HMPV and one or more non-
HMPV pathogens, in a subject susceptible to infection. Recombinant HMPV as disclosed herein are

25   capable of eliciting a mono- or poly-specific immune response in an infected mammalian host, yet are
sufficiently attenuated so as to not cause unacceptable symptoms of disease in the immunized host.
The attenuated viruses, including complete viruses and subviral particles, can be present in a cell
culture supernatant, isolated from the culture, or partially or completely purified. The virus can also
be lyophilized, and can be combined with a variety of other components for storage or delivery to a

30   host, as desired.

        Immunogenic compositions are also disclosed herein that include a physiologically
acceptable carrier and/or adjuvant and an isolated attenuated recombinant HMPV virus. In one
embodiment, the immunogenic composition includes a recombinant HMPV having at least one, at
least two, or more attenuating mutations or other nucleotide modifications that specify a suitable

35   balance of attenuation and immunogenicity. In specific examples, the immunogenic composition can
be formulated in a dose of 10^3 to 10^7 PFU of attenuated virus. The immunogenic composition can
include an attenuated recombinant HMPV that elicits an immune response against a single HMPV

strain or against multiple HMPV strains or serotypes or other pathogens such as RSV and/or HPIV.
In this regard, recombinant HMPV can be combined in formulations with other HMPV strains, or
with other candidate viruses such as a live attenuated RSV. Methods are also disclosed herein for
stimulating the immune system of a mammalian subject to elicit an immune response against one or

5   more HMPVs, or against HMPV and a non-HMPV pathogen. Thus, a method is provided herein for
inducing an immune response against a single HMPV, against multiple HMPVs, or against one or
more HMPVs and a non-HMPV pathogen such as RSV.

            The disclosed recombinant HMPVs can be used to elicit a monospecific immune response or
a polyspecific immune response against multiple HMPVs, or against one or more HMPVs and a non-

10   HMPV pathogen. Alternatively, recombinant HMPV having different immunogenic characteristics
can be combined in a mixture or administered separately in a coordinated treatment protocol to elicit
an immune response against one HMPV, against multiple HMPVs, or against one or more HMPVs
and a non-HMPV pathogen such as RSV. In one example, the immunogenic compositions are
administered to the upper respiratory tract, for example, by spray, droplet or aerosol.

15   An operable set of viral open reading frames (ORFs) sufficient to direct viral transcription
and RNA replication are disclosed herein. Diagnostic assays using this set of ORFs are provided that
determine viral activities based either on infectious virus or on the expression of isolated
polynucleotide molecules encoding viral sequences. In one example, an infectious HMPV
recombinant virus is disclosed herein that expresses a detectable marker or label, such as a

20   recombinant HMPV expressing the jellyfish green fluorescent protein (GFP). These and related tools
are effective, for example, to determine and characterize HMPV infection *in vitro* and *in vivo*. In one
exemplary embodiment, recombinant HMPV expressing a detectable label can be utilized in assays
and related compositions for the detection of HMPV-neutralizing antibodies in biological specimens
(for example, serum of patients at risk of HMPV infection or presenting with respiratory symptoms).

25   In additional embodiments the recombinant HMPV expressing a detectable label can be used to
screen compounds for antiviral activity.

            Unless otherwise noted, technical terms are used according to conventional usage.
Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes VII*,
published by Oxford University Press, 2000 (ISBN 019879276X); Kendrew *et al.* (eds.), *The*

30   *Encyclopedia of Molecular Biology*, published by Blackwell Publishers, 1994 (ISBN 0632021829);
and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk
Reference*, published by Wiley, John & Sons, Inc., 1995 (ISBN 0471186341); and other similar
references.

            As used herein, the singular terms "a," "an," and "the" include plural referents unless context

35   clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context
clearly indicates otherwise. Also, as used herein, the term "comprises" means "includes." Hence
"comprising A or B" means including A, B, or A and B. It is further to be understood that all base

sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids

or polypeptides are approximate, and are provided for description. Although methods and materials

similar or equivalent to those described herein can be used in the practice or testing of the present

invention, suitable methods and materials are described below. All publications, patent applications,

5   patents, and other references mentioned herein are incorporated by reference in their entirety. In case

of conflict, the present specification, including explanations of terms, will control. In addition, the

materials, methods, and examples are illustrative only and not intended to be limiting.

10   As described above, methods and compositions for the production and use of novel,

recombinant HMPVs are provided herein. The recombinant HMPVs are infectious and immunogenic

in humans and other mammals and are useful as immunogenic compositions to produce immune

responses against one or more HMPVs or other pneumoviruses. Thus, the recombinant HMPVs can

be used to produce an immune response to, for example, RSV and/or one or more HMPV or other

15   pneumoviral strains, serotypes, or subgroups. Chimeric HMPVs are provided herein that can be used

to elicit an immune response against a selected HMPV and one or more additional pathogens, for

example against multiple HMPVs or against a HMPV and a non-HMPV virus such as RSV, or

parainfluenza virus (PIV). The immune response elicited can involve either or both humoral and/or

cell mediated responses. The HMPVs can be attenuated to yield a desired balance of attenuation and

20   immunogenicity.

       Methods are provided herein for designing and producing attenuated, HMPVs that are useful

as agents for eliciting a desired immune response against HMPV and other pathogens. Thus, using

the methods disclosed herein, recombinant HMPVs can be produced that have a defined genome

sequence and predictable characteristics.

25   An exemplary recombinant HMPV (rHMPV) includes a recombinant HMPV genome or

antigenome and encodes (or the antisense of which encodes) HMPV major nucleocapsid (N) protein,

nucleocapsid phosphoprotein (P), and large polymerase protein (L) (see FIG. 1 and exemplary

sequence information provided in FIG. 37). In additional embodiments, the rHMPV can incorporate

a recombinant HMPV genome or antigenome, N, P, and L proteins, and a HMPV M2-1 protein. In

30   further embodiments, one or more of the N, P, L, and/or M2-1 proteins can be a mutant protein or

partial protein, or include a corresponding homologous protein or protein fragment of a heterologous

HMPV or non-HMPV virus such as APV. One or more additional HMPV proteins can be

coexpressed with the recombinant HMPV genome or antigenome, in various combinations, to

provide a range of infectious viruses. As used herein the term "recombinant HMPV" or "rHMPV"

35   includes recombinantly produced subviral particles lacking one or more non-essential viral

components, complete viruses having all native viral components, and viruses containing

supernumerary proteins, antigenic determinants or other additional components. A recombinant virus is produced entirely from cloned cDNA.

As set forth in the Examples below, a complete consensus sequence was determined for the genomic RNA of an exemplary wild type strain of HMPV. The sequence thus identified was used to

5   generate a full-length antigenomic cDNA and to recover a wild type rHMPV. The biological properties of rHMPV *in vitro* and *in vivo* demonstrates that the exemplary rHMPV sequence corresponds to a wild type virus. This critical finding demonstrates that the recombinant HMPV sequence disclosed herein is that of an authentic wild type virus. This rHMPV serves as a novel substrate for recombinant introduction of attenuating mutations for the generation of live-attenuated

10   HMPV and HMPV-based chimeric and vectors that can be used for immunogenic compositions.

As disclosed herein, infectious recombinant HMPVs can be produced by a recombinant coexpression system that permits introduction of defined changes into the recombinant HMPV and provides for the generation, with high frequency and fidelity, of HMPV having a defined genome sequence. These-modifications are useful in a wide-variety of applications, including the

15   development of live attenuated viral strains bearing predetermined, defined attenuating mutations. Infectious HMPV can be produced by intracellular or cell-free coexpression of one or more isolated polynucleotide molecules that encode the HMPV genome or antigenome RNA, together with one or more polynucleotides encoding viral proteins to generate a transcribing, replicating nucleocapsid. An "infectious virus" is a viral particle that has the ability to deliver its genome to the cytoplasm of a

20   host cell.

The cDNAs encoding a HMPV genome or antigenome are constructed for intracellular or *in vitro* coexpression with the selected viral proteins to form infectious HMPV. A "HMPV antigenome" is an isolated positive-sense polynucleotide molecule which serves as a template for synthesis of progeny HMPV genome. In one embodiment a cDNA is constructed which is a positive-

25   sense version of the HMPV genome that corresponds to the replicative intermediate RNA, or antigenome. This minimizes the possibility of hybridizing with positive-sense transcripts of complementing sequences encoding proteins necessary to generate a transcribing, replicating nucleocapsid. A "replication competent virus" is a viral particle capable of initiating an infection that produces viral progeny.

30   In some embodiments the genome or antigenome of a recombinant HMPV contains only those genes or portions thereof necessary to render the viral or subviral particles encoded thereby infectious. Further, the genes or portions thereof may be provided by more than one polynucleotide molecule, that is, a gene may be provided by complementation or the like from a separate nucleotide molecule. In other embodiments, the HMPV genome or antigenome encodes all functions necessary

35   for viral growth, replication, and infection without the participation of a helper virus or viral function provided by a plasmid or helper cell line.

A "recombinant HMPV particle" is a HMPV or HMPV-like viral or subviral particle derived directly or indirectly from a recombinant expression system or propagated from virus or subviral particles produced there from. The recombinant expression system can employ a recombinant expression vector which includes an operably linked transcriptional unit comprising an assembly of at

5   least a genetic element or elements having a regulatory role in HMPV gene expression, for example, a promoter, a structural or coding sequence which is transcribed into HMPV RNA, and appropriate transcription initiation and termination sequences. A first nucleic acid sequence is "operably linked" with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence.

10   To produce infectious HMPV from a cDNA-expressed HMPV genome or antigenome, the genome or antigenome is coexpressed with those HMPV or heterologous viral proteins necessary to produce a nucleocapsid capable of RNA replication, and render progeny nucleocapsids competent for both RNA replication and transcription. Transcription by the genome nucleocapsid provides the other HMPV proteins and initiates a productive infection. Alternatively, additional HMPV proteins

15   useful for a productive infection can be supplied by coexpression.

In certain embodiments, complementing sequences encoding proteins necessary to generate a transcribing, replicating HMPV nucleocapsid are provided by one or more helper viruses. Such helper viruses can be wild type or mutant. In one embodiment, the helper virus can be distinguished phenotypically from the virus encoded by the HMPV cDNA. For example, it may be desirable to

20   provide monoclonal antibodies that react immunologically with the helper virus but not the virus encoded by the HMPV cDNA. Such antibodies can be neutralizing antibodies. In some embodiments, the antibodies can be used in affinity chromatography to separate the helper virus from the recombinant virus. To aid the procurement of such antibodies, mutations can be introduced into the HMPV cDNA to provide antigenic diversity from the helper virus, such as in the HMPV

25   glycoprotein genes.

Expression of the HMPV genome or antigenome and proteins from transfected plasmids can be achieved, for example, by each cDNA being under the control of a selected promoter (for example, for T7 RNA polymerase), which in turn is supplied by infection, transfection or transduction with a suitable expression system (for example, for the T7 RNA polymerase, such as a

30   vaccinia virus MVA strain recombinant which expresses the T7 RNA polymerase, as described by Wyatt et al., Virology 210:202-205, 1995). The viral proteins, and/or T7 RNA polymerase, can also be provided by transformed mammalian cells (see Buchholz et al., J. Virol. 73:251-259. 1999) or by transfection of preformed mRNA or protein.

A HMPV genome or antigenome can be constructed for use by, for example, assembling

35   cloned cDNA segments, representing in aggregate the complete genome or antigenome, by PCR or the like (described in, for example, U.S. Patent Nos. 4,683,195 and 4,683,202, and PCR Protocols: A Guide to Methods and Applications, Innis et al., eds., Academic Press, San Diego, 1990) of reverse-

-25-

transcribed copies of HMPV mRNA or genome RNA. For example, a first construct can be generated which includes cDNAs containing the left hand end of the antigenome, spanning from an appropriate promoter (for example, T7 RNA polymerase promoter) and assembled in an appropriate expression vector, such as a plasmid, cosmid, phage, or DNA virus vector. The vector can be

5   modified by mutagenesis and/or insertion of a synthetic polylinker containing unique restriction sites designed to facilitate assembly. For ease of preparation the N, P, L and other desired HMPV proteins can be assembled in one or more separate vectors. The right hand end of the antigenome plasmid may contain additional sequences as desired, such as a flanking ribozyme and single or tandem T7 transcriptional terminators. The ribozyme can be hammerhead type, which would yield a 3' end

10   containing a single nonviral nucleotide, or can be any of the other suitable ribozymes such as that of hepatitis delta virus (Perrotta et al., Nature 350:434-436, 1991) that would yield a 3' end free of non-PIV nucleotides.

Alternative means to construct cDNA encoding the HMPV genome or antigenome include RT-PCR using different PCR conditions (for example, as described in Cheng et al., Proc. Natl. Acad.

15   Sci. USA 91:5695-5699, 1994) to reduce the number of subunit cDNA components to as few as one or two pieces. In other embodiments different promoters can be used (for example, T3, SPQ or different ribozymes, such as that of a hammerhead variety. Different DNA vectors (for example, cosmids) can be used for propagation to better accommodate the larger size genome or antigenome.

By "infectious clone" or "infectious cDNA" of HMPV is meant cDNA or its product,

20   synthetic or otherwise, as well as RNA capable of being directly incorporated into infectious virions which can be transcribed into genomic or antigenomic HMPV RNA that can serve as a template to produce the genome of infectious HMPV viral or subviral particles. As noted above, defined mutations can be introduced into an infectious HMPV clone by a variety of conventional techniques (for example, site-directed mutagenesis) into a cDNA copy of the genome or antigenome. The use of

25   genomic or antigenomic cDNA subfragments to assemble a complete genome or antigenome cDNA as described herein has the advantage that each region can be manipulated separately, where small cDNA constructs provide for better ease of manipulation than large cDNA constructs, and then readily assembled into a complete cDNA.

Isolated polynucleotides (for example, cDNA) encoding the HMPV genome or antigenome

30   can be introduced into cells that can support a productive HMPV infection by transfection, electroporation, mechanical insertion, transduction or the like. Exemplary cells of use are HEp-2, FRhL-DBS2, LLC-MK2, MRC-5, baby hamster kidney (BHK), and Vero cells. Isolated polynucleotide sequences can be introduced into cultured cells by, for example, calcium phosphate-mediated transfection (Wigler et al., Cell 14:725, 1978; Corsaro et al., Somatic Cell Genetics 7:603,

35   1981; Graham et al., Virology 52:456, 1973, electroporation (Neumann et al., EMBO J. 1:841-845, 1982), DEAE-dextran mediated transfection (Ausubel et al., (ed.) Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987), cationic lipid-mediated transfection (Hawley-Nelson

et al., Focus 15:73-79, 1993) or a commercially available transfection regent, for example, Lipofectamine-2000 (Invitrogen, Carlsbad, CA) or the like.

By providing infectious clones of HMPV, a wide range of alterations can be recombinantly produced within the HMPV genome (or antigenome), yielding defined mutations that specify desired

5   phenotypic changes. The compositions and methods disclosed herein for producing recombinant HMPV permit ready detailed analysis and manipulation of HMPV molecular biology and pathogenic mechanisms using, for example, defined mutations to alter the function or expression of selected HMPV proteins. Using these methods and compositions, one can readily distinguish mutations responsible for desired phenotypic changes from silent incidental mutations, and select phenotype-

10   specific mutations for incorporation into a recombinant HMPV genome or antigenome. In this context, a variety of nucleotide insertions, deletions, substitutions, and rearrangements can be made in the HMPV genome or antigenome during or after construction of the cDNA. For example, specific desired nucleotide sequences can be synthesized and inserted at appropriate regions in the cDNA using convenient restriction enzyme-sites. Alternatively, such techniques as site-specific

15   mutagenesis, alanine scanning mutagenesis, PCR mutagenesis, or other such techniques well known in the art can be used to introduce mutations into the cDNA.

Recombinant modifications of HMPV can be directed toward the production of improved candidate viruses, for example, to enhance viral attenuation and immunogenicity, to ablate epitopes associated with undesirable immunopathology, to accommodate antigenic drift, etc. To achieve these

20   and other objectives, the compositions and methods disclosed herein allow for a wide variety of modifications to be introduced into a HMPV genome or antigenome for incorporation into infectious, recombinant HMPV. For example, foreign genes or gene segments encoding antigenic determinants (for example, protective antigens or immunogenic epitopes) can be added within a HMPV clone to generate recombinant HMPVs capable of inducing immunity to both HMPV and another virus or

25   pathogenic agent from which the antigenic determinants was/were derived. Alternatively, foreign genes can be inserted, in whole or in part, encoding modulators of the immune system, such as cytokines, to enhance immunogenicity of a candidate virus. Other mutations that can be included within HMPV clones are, for example, substitution of heterologous genes or gene segments (for example, a gene segment encoding a cytoplasmic tail of a glycoprotein gene) with a counterpart gene

30   or gene segment in a HMPV clone. Alternatively, the relative order of genes within a HMPV clone can be changed, a HMPV genome promoter or other regulatory element can be replaced with its antigenome counterpart, or selected HMPV genes rendered non-functional (for example, by functional ablation involving introduction of a stop codon to prevent expression of the gene). In addition, the codon selection of genes such as those encoding the major protective antigens can be

35   modified to improve the efficiency of translation. Other modifications in a HMPV clone can be made to facilitate manipulations, such as the insertion of unique restriction sites in various non-coding or

coding regions of the HMPV genome or antigenome. In addition, nontranslated gene sequences or intergenic regions can be shortened or removed to increase capacity for inserting foreign sequences.

As noted above, it is often desirable to adjust the phenotype of recombinant HMPVs for use by introducing additional mutations that attenuate the virus, affect the virulence of the virus, or

5   otherwise alter the phenotype of the recombinant virus. One of skill in the art can readily identify methods and procedures for mutagenizing, isolating and characterizing HMPV to obtain attenuated mutant strains (for example, temperature sensitive (*ts*), cold passaged (*cp*), cold-adapted (*ca*), small plaque (*sp*) and host-range restricted (*hr*) mutant strains) and for identifying the genetic changes that specify the attenuated phenotype (see, for example, Durbin *et al.*, Virology 235:323-332, 1997; U.S.

10   Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Patent Application Serial No. 09/458,813, filed December 10, 1999; U.S. Patent Application Serial No. 09/459,062, filed December 10, 1999; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078), and U.S. Provisional Application No. 60/059,385, filed September 19, 1997). Thus, methods are provided herein for determining replication,

15   immunogenicity, genetic stability and immunogenic efficacy of biologically derived and recombinantly produced attenuated HMPVs in accepted model systems reasonably correlative of human activity, including hamster or rodent and non-human primate model systems.

In additional embodiments, rHMPV candidates are constructed by introduction of nucleotide or amino acid point mutations that confer attenuation or other desired phenotypes. Such mutations

20   can involve substitution of one or more nucleotides or amino acids at a given locus, or can involve small deletions in which one or more nucleotides or amino acids at a given locus are deleted. These mutations are termed "point mutations" here to denote that each particular mutation is circumscribed. For example, a typical mutation can involve changing a single amino acid by substituting 1, 2 or 3 nucleotides in the corresponding codon. As another example, the three nucleotides might be deleted

25   altogether, resulting in the deletion of a single amino acid in the encoded protein. Point mutations can be identified empirically, such as by systematically replacing charged amino acids in one or more of the HMPV proteins with ones that are uncharged, for example, replacing aspartate, glutamate, lysine or arginine with alanine.

Alternatively, attenuating amino acid substitutions and other mutations can be devised using

30   existing mononegavirus mutations as a guide, for example mutations in a different "biologically derived" HMPV, non-human pneumovirus, such as an APV, or non-HMPV virus, such as a RSV or PIV. By "biologically derived" is meant any virus not produced by recombinant DNA methods. Thus, biologically derived HMPV include all naturally occurring HMPVs, including, for example, naturally occurring HMPV having a wild type genomic sequence and HMPV having allelic or mutant

35   genomic variations from a reference wild type HMPV sequence, for example, HMPV having a mutation specifying an attenuated phenotype. Likewise, biologically derived HMPV include HMPV mutants derived from a parental HMPV by, inter alia, artificial mutagenesis and selection procedures

not involving direct recombinant DNA manipulation. Attenuating mutations in biologically derived HMPV and other nonsegmented negative stranded RNA viruses for incorporation within recombinant HMPV can occur naturally or can be introduced into wild type HMPV strains and thereafter identified and characterized by well known mutagenesis and analytic procedures. For example,

5   incompletely attenuated parental HMPV or other heterologous viral mutant strains can be produced by chemical mutagenesis during virus growth in cell cultures to which a chemical mutagen has been added, by selection of virus that has been subjected to passage at suboptimal temperatures in order to introduce growth restriction mutations, or by selection of a mutagenized virus that produces small plaques (sp) in cell culture. In addition, known mutations in existing mononegaviruses that have

10   been produced by recombinant methods also can serve as a guide for devising desired mutations in HMPV. Such known mutations in heterologous viruses can be ones that had been deliberately introduced by directed mutagenesis, or can be ones that arose spontaneously during the process of DNA manipulation and virus recovery and propagation, or can ones that arose in recombinantly derived viruses that were exposed subsequently to mutagenic agents or passaged in a manner

15   designed to favor phenotypic changes, such as described above for generating mutations in biologically derived viruses.

In certain embodiments, the HMPV genome or antigenome is recombinantly modified to incorporate an attenuating mutation at an amino acid position corresponding to an amino acid position of an attenuating mutation identified in a heterologous, mutant nonsegmented negative

20   stranded RNA virus. The virus can be either biologically derived or of recombinant origin. Based on routine sequence alignments and other analyses, mutations previously identified in a heterologous HMPV or non-HMPV virus are mapped to a corresponding position in HMPV for "transfer" (that is, introduction of an identical, conservative or non-conservative mutation, potentially including a substitution, deletion or insertion, at a homologous or corresponding position identified by the

25   alignment) into recombinant HMPV. In many cases, an alignment of the two sequences of interest is sufficient to identify the corresponding residue in HMPV, particularly if the percent amino acid identity is substantial (for example, approximately 35-40% identity or greater) or high (for example, 70% identity or greater, such as at least about 80%, 90%, 95%, or 99% identity). In other cases, particularly when the amino acid relatedness is not substantial (for example, less than approximately

30   35% identity), additional heterologous related viruses can be included in the alignment in order to obtain reliably identify conserved residues that serve as markers to identify corresponding positions. In one embodiment. the percentage of sequence identity associated with the terms "substantial" or "high" or "not high" are used herein in the context of comparison of heterologous viruses from different serotypes or taxonomic groups.

35   A large assemblage of such candidate mutations are available for transfer into rHMPV of the current disclosure (see, for example, (Durbin et al., Virology 235:323-332, 1997; Skiadopoulos et al., J. Virol. 72:1762-1768, 1998; Skiadopoulos et al., J. Virol. 73:1374-1381, 1999; Skiadopoulos et al.,

Virology 260:125-35, 1999; Durbin *et al.*, Virology 261:319-30, 1999; Newman *et al.*, Virus Genes

24:77-92, 2002; Feller *et al.*, Virology 276:190-201, 2000; U.S. Patent Application Serial No.

09/083,793, filed May 22, 1998; U.S. Patent Application Serial No. 09/458,813, filed December 10,

1999; U.S. Patent Application Serial No. 09/459,062, filed December 10, 1999; U.S. Provisional

5   Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO

98/53078); U.S. Provisional Application No. 60/059,385, filed September 19, 1997; U.S. Patent

Application No. 10/302,547, filed November 21, 2002 and priority U.S. Provisional Application No.

60/331,961, filed November 21, 2001; and U.S. Provisional Application No. 60/412,053, filed

September 18, 2002).

10   Within these embodiments, it is often desired to modify the recipient recombinant HMPV

genome or antigenome to encode an alteration at the subject site of mutation that corresponds

conservatively to the alteration identified in the heterologous mutant virus. For example, if an amino

acid substitution marks a site of mutation in the mutant virus compared to the corresponding wild

type sequence, then a similar substitution can be engineered at the corresponding residues in the

15   recombinant HMPV. In one example, the substitution will specify an identical or conservative amino

acid to the substitute residue present in the mutant viral protein (see below for a description of

conservative substations). However, it is also possible to alter the native amino acid residue at the

site of mutation non-conservatively with respect to the substitute residue in the mutant protein (for

example, by using any other amino acid to disrupt or impair the function of the wild type residue).

20   Negative stranded RNA viruses from which exemplary mutations are identified and transferred into a

recombinant HMPV of the current disclosure include heterologous strains of HMPV, other non-

HMPV pneumoviruses (for example, human RSV, bovine RSV, APV, pneumonia virus of mice

(PVM)), PIVs (for example, HPIV1, HPIV2, HPIV3, BPIV3, and MPIV1), Newcastle disease virus

(NDV), simian virus 5 (SV5), measles virus (MeV), rinderpest virus, canine distemper virus (CDV),

25   rabies virus (RaV), and vesicular stomatitis virus (VSV), among others.

As depicted in Figures 26A-26F, one or more corresponding sites of mutation that specify a

desired phenotypic change in a heterologous virus (for example, when indicated wild type residues at

the designated positions is/are altered, for example, by substitution) is identified by conventional

sequence alignment. The corresponding target sites for mutation (see, for example, boxed sites in

30   Figures 26A-26F) in HMPV is thereby mapped for transfer of the mutation into a recombinant

HMPV to yield attenuation or other desired phenotypic changes. More specifically, corresponding

amino acids between the compared heterologous mutant and HMPV sequences represent target sites

for identical or conservative transfer (for example, by recombinant engineering involving site-

directed mutagenesis of a HMPV antigenomic cDNA) of the subject mutation into a recombinant

35   HMPV. In some embodiments, attenuating and other desired mutations identified in one negative

stranded RNA virus are thereby targeted for transfer (for example, to be copied identically or

conservatively by substitution mutagenesis) into a corresponding position within the genome or

antigenome of a recombinant HMPV of the current disclosure. Related methods for rational design

of other recombinant mutant RNA viruses are described, for example, in International Application

No. PCT/US00/09695, filed April 12, 2000, published as WO 00/61737 on 10/19/00 corresponding to

U.S. National Phase application 09/958,292, filed on 01/08/02, and claiming priority to U.S.

5   Provisional Patent Application Serial No. 60/129,006, filed on April 13, 1999. Additional description

pertaining to this aspect of the current disclosure is provided in Newman *et al.*, Virus Genes 24:77-

92, 2002; Feller *et al.*, Virology 10;276:190-201, 2000; Skiadopoulos *et al.*, Virology 260:125-35,

1999; and Durbin *et al.*, Virology 261:319-30, 1999.

In one exemplary embodiment, amino acid sequence alignments are made between the L

10   gene of HMPV and the L genes of other mononegaviruses for which one or more attenuating

substitutions in the L protein have been identified. This embodiment of the current disclosure is

illustrated in FIGS. 26A-26F, which depict sequence alignments of various segments of the putative

L protein of HMPV with corresponding segments of L proteins of a number of other

mononegaviruses in which attenuating mutations have been mapped. As exemplified in FIGS. 26A-

15   26F, various mutations identified in the L gene of a heterologous negative stranded RNA virus can be

incorporated into recombinant HMPV to yield attenuation or other desired phenotypic changes.

These figures provide exemplary, partial L gene sequence alignments between an HMPV wild type

virus and the indicated viruses (including as examples, a different HMPV, RSV, HPIV1 HPIV3, and

BPIV3). The examples of attenuating mutations include ones identified in viruses of biological or

20   recombinant origin. The alignments are employed to identify regions containing known attenuating

mutations in the heterologous virus.

FIGS. 26A-26F illustrate the identification and mapping of various exemplary attenuating

mutations in the L gene for transfer into rHMPV (note that the numbers at the left indicate the

positional reference number of the first residue in the partial sequence shown, and the numbers at the

25   right indicate the positional reference number of the terminal residue in the partial sequence). FIG.

26A shows a Phenylalanine-521 to Leucine (F521L) substitution in the L protein specified by a

mutation in the L gene of an RSV cold-passage temperature-sensitive (cpts) mutant cpts530 (Juhasz

et al., J. Virol. 71:5814-9, 1999). Also shown is an attenuating double mutation of Arginine-588-

Alanine (R588A) and Aspartate-589-Alanine (D589A) that was originally developed in RSV (Tang *et

30   al.*, Virology 302:207-16, 2002). FIG. 26B shows the positions of attenuating Tyrosine-942-

Histidine (Y942H) and Leucine-992-Tyrosine (L992Y) mutations identified in the PIV3 cp45 vaccine

candidate (Skiadopoulos *et al.* J. Virol. 72:31762-8, 1998; U.S. Patent Application No. 10/302,547,

filed November 21, 2002 and priority U.S. Provisional Application No. 60/331,961, filed November

21, 2001; and U.S. Provisional Application No. 60/412,053, filed September 18, 2002). FIG. 26C

35   shows the positions of the attenuating Isoleucine-1103-Valine (I1103V) mutation originally identified

in BPIV3 (Haller *et al.*, Virology 288:342-50, 2001), the attenuating Threonine-1558-Isoleucine

(T1558I) mutation identified in PIV3 cp45 (Skiadopoulos *et al.*, J. Virol. 72:31762-8, 1998), and the

Cysteine-319-Tyrosine (C319Y) mutation identified the attenuated cpRSV vaccine candidate (Connors et al., Virology 208:478-84, 1995). FIG. 26D shows the positions of the attenuating Glutamine-831-Leucine (Q831L) mutation identified in cpts248 RSV (Firestone et al., Virology 225:419-22, 1996), the attenuating Methionine-1169-Valine (M1169V) mutation identified in the

5   RSV mutant cpts530/109 (Juhasz et al., J. Virol. 73:5176-80), the Aspartate-1183-Glutamate (D1183) mutation identified in the RSV mutant cpts248/404 (Firestone et al., Virology 225:419-22, 1996), and the combination of six point mutations that constitute the attenuating C9 cluster characterized in recombinant RSV (Tang et al., Virology 302:207-16, 2002): Aspartate-1187-Alaine (D1187A), Lysine-1188-Alanine (K1188A), Arginine-1189-Alanine (R1189A), Glutamate-1190-

10   Alanine (E1190A), Glutamate-1208-Alanine (E1208A), and Arginine-1209-Alanine (R1209A). FIG. 26E shows the position of the attenuating Tyrosine-1321-Asparagine (Y1321N) mutation of RSV cpts530/1030 (Whitehead et al., J. Virol. 73:871-7, 1999), and the Histidine-1690-Tyrosine (H1690Y) mutation of the attenuated cpRSV derivative (Connors et al., Virology 208, 478-84, 1995). FIG. 26F shows the positions of an attenuating Asparagine-43-Isoleucine (N43I) mutation identified

15   in the cpts RSV mutant 248/955, and the attenuating Threonine-1711-Isoleucine (T1711I) mutation characterized in the chimeric virus rHPIV3-L$_B$, consisting of HPIV3 in which the L gene was replaced by that of BPIV3 (Skiadopoulos et al., J. Virol. 77:1141-8, 2003).

The foregoing alignments show, for example, that the F521 residue in the RSV sequence is exactly conserved among most of the other mononegaviruses examined (23 out of 24

20   paramyxoviruses and 1 out of 3 rhabdoviruses) and, in particular, is present in HMPV. It should be noted that the amino acid position of the corresponding residue is not the same in each L protein, reflecting differences in length as well as small deletions and insertions elsewhere in the various individual molecules. Thus, the corresponding residue is F456 in HMPV. From this comparative mapping analysis, the F456 residue in HMPV is identified as a target for either an identical,

25   conservative, or even non-conservative amino acid substitution (for example, substitution of the F456 residue by a leucine, or by a conservative or non-conservative amino acid as compared to leucine). The same alignment (FIG. 26A) shows that the D589 assignment is also exactly conserved in each of sequences. The R588 assignment is not conserved in each, but it is represented by the homologous assignment of Lysine in HMPV. With respect to the attenuating Tyrosine-942-Histidine (Y942H)

30   and Leucine-992-Tyrosine (L992Y) mutations of PIV3 cp45, in both of these instances the exact amino acid assignment was not conserved, but the presence of various highly-conserved residues throughout the alignment show that these positions correspond (Figure 26B). Positional correspondence in this context indicates that the aligned, corresponding target site for mutation (whether it is occupied by an amino acid that is identical, conservative or divergent in structural

35   character compared the corresponding residue in the wild type (for example, a non-attenuated parent) sequence of the heterologous virus) will often yield an attenuation or other desired phenotype in a rHMPV upon mutation from the HMPV wild type sequence to a different residue (that is, to a residue

identity that is the same, conservative, or even distinct structurally in comparison to the corresponding residue in the heterologous mutant).

In addition to the foregoing L gene mutations, two temperature sensitive (ts) mutations that were reported in the P protein of recombinant RSV, namely Glycine-172-Serine and Glutamate-176-

5   Glycine (Lu et al., J. Virol. 76:2871-80, 2002), can be transferred to yield a predicted attenuation phenotype in a rHMPV P, by altering the corresponding one or both of the HMPV residues Glycine-213 and Glutamate-217, respectively. Likewise, a mutation in the N protein of the attenuated derivative cpRSV, specifying a Valine-267-Isoleucine substitution (Connors et al. Virology 208:478-84, 1996), maps to Serine-268 in HMPV, whereby an Ile or other, conservative or non-conservative,

10   substitution of this residue in a rHMPV can yield useful candidates for identification of additional attenuated derivatives. In the same manner, two mutations in the F protein of cpRSV, namely Glutamate-218-Alanine and Threonine-523-Isoleucine (Connors et al. Virology 208:478-84, 1996), correspond to Lysine-188 and Threonine-489, respectively, in the HMPV F protein, which therefore represent target sites for introduction of an attenuating amino acid substitution in rHMPV.

15   In other embodiments, mutations in a cis-acting regulatory sequence or other non-coding sequence are identified in a heterologous virus (including biologically or otherwise derived mutant HMPV of the same or different strain, subtype, species, RSV, PIV, APV, etc.) and incorporated in a rHMPV of the current disclosure. For example, the identification of the GS and GE motifs identifies these as targets for mutagenesis. The short, circumscribed nature of these signals is amenable to a

20   systematic evaluation of the effects of mutations at each position. Mutations in RNA signals such as these can be readily assayed in a mini-replicon system as well as in complete virus.

FIG. 28 shows an example of transferring a mutation that involves a cis-acting RNA signal. A potent attenuating point mutation identified for the cpts 248/404 derivative of RSV involves a point mutation in the GS signal of the M2 gene: specifically, the T residue (positive sense) at position nine

25   was changed to C (see, for example, Whitehead et al., Virology 247:232-9, 1998). In the proposed GS signal of HMPV, a T residue at position nine is conserved in each of the proposed HMPV genes (FIG. 28). This substitution can be made in any of the HMPV genes.

In accordance with the foregoing description, a large panel of desired mutations identified in heterologous viruses can be "transferred" into a rHMPV, even in cases of low sequence relatedness.

30   In these embodiments, there is not a strict requirement that the corresponding wild type or final mutant amino acid assignment be identical to that of the heterologous virus. Also, rather than an amino acid substitution, it is possible to delete one or several amino acids or, alternatively, insert one or several amino acids. In designing substitution mutations for HMPV, a useful strategy is to evaluate all possible alternative amino acid assignments at this position. This is a useful method for

35   evaluating the full range of possible attenuation phenotypes involving this position, and can produce mutants exhibiting a range of temperature sensitivity and attenuation (see, for example, U.S. Patent Application No. 10/302,547, filed November 21, 2002 and priority U.S. Provisional Application No.

60/331,961, filed November 21, 2001; and U.S. Provisional Application No. 60/412,053, filed

September 18, 2002). In addition, the choice of codon for substitution can be made to involve the

greatest number of nucleotide differences relative to both the wild type and to any possible alternative

amino acid assignments that are found to not yield attenuated phenotypes. This has the effect of

5   reducing the probability of reversion or mutation to lose the attenuated phenotype. Specifically, it is

estimated that the frequency of reversion at any single nucleotide position is 10^{-4} to 10^{-5}, and hence

the frequency of reversion/mutation at two nucleotide positions is 10^{-8} to 10^{-10}, and the frequency of

reversion/mutation involving three nucleotides is 10^{-12} to 10^{-15}. Thus, point mutations involving

multiple nucleotide substitutions can greatly increase genetic and phenotypic stability. Also, the

10   mutation can be designed to involve deletion of a single amino acid or more than one amino acid,

which can reduce the possibility of reversion.

It should be noted that the mutations illustrated in FIGS. 26A-26F are only exemplary by

virtue that the depicted mapping exercise was limited to examples involving amino acid changes in

the L protein, whereas the same strategy can be applied in accordance with the tools and description

15   provided herein to transfer attenuating mutations involving any viral protein. For example, two *ts*

mutations that were developed in the P protein of recombinant RSV, namely Glycine-172-Serine and

Glutamate-176-Glycine (Lu *et al.*, J. Virol. 76:2871-80, 2002), correspond to HMPV P residues

Glycine-213 and Glutamate-217, respectively. A mutation in the N protein of the attenuated

derivative *cp*RSV, namely Valine-267-Isoleucine (Connors *et al.* Virology 208:478-84, 1996),

20   corresponds to Serine-268 in HMPV. Also, two mutations in the F protein of *cp*RSV, namely

Glutamate-218-Alanine and Threonine-523-Isoleucine (Connors *et al.* Virology 208:478-84, 1996),

correspond to Lysine-188 and Threonine-489, respectively, in the HMPV F protein. Thus, any known

mutation having a desirable phenotypic property present in any nucleotide or amino acid sequence is

a useful candidate for incorporation in rHMPV by mutation of corresponding residues in a rHMPV

25   RNA or protein employing the novel tools and methods of the current disclosure.

Another method for devising attenuating or otherwise desirable amino acid substitution

mutations involve "charge-to-alanine" or "alanine scanning" mutagenesis where, for example, one or

more charged amino acid residues are each changed to alanine residues (Wertman *et al.*, Genetics

132:337-50, 1992; Hassett and Condit, Proc. Natl. Acad. Sci. USA 91:4554-58, 1994; Tang *et al.*,

30   Virology 302:207-16, 2002. A typical target is a pair of residues, but residues can be changed singly

or in larger combinations. It is often useful to systematically change ("scan") residues across an

encoded protein. Any protein can be a target, although the L protein is the most common locus of

mutations in attenuated mononegaviruses. Alternatively, one advantage of this strategy is that one can

target proteins that are not represented in available mutants. This strategy can be readily addressed

35   with the methods disclosed herein. In particular, the minireplicon system offers a method for

screening mutations in the N, P, L and M2-1 proteins. In that strategy, mutations are introduced into

the appropriate support plasmid, such as the one encoding the L protein, and evaluated for the ability

to direct the replication and transcription of a minigenome containing a reporter gene. For example, the assay can be done in replica at 32°C and 37°C (or any temperature in the range of 35°C -41°C) to identify ts mutations that operate at the lower, permissive temperature and are inhibited at the higher temperature. This offers a more rapid and technically simple method for performing an initial

5   screening, after which appropriate mutations can be introduced into complete recombinant virus.

Thus, the methods disclosed herein can be used to develop mutations that confer improved properties to HMPV for the purposes of developing an immunogenic composition or for other purposes, such as purifying viral proteins. Mutations can be optimized, such as by evaluating all possible amino acid or nucleotide assignments at a given position, or by making amino acid changes

10   involve codon choices that will be less likely to revert to wild type or to an alternative assignment that yields a wild type phenotype. Other changes can be made that fundamentally alter the viral backbone, such as changes in gene order or movement of major protective antigen genes to a promoter-proximal position. Importantly, these various modifications can be introduced into rHMPV in combination to develop viruses for use in immunogenic compositions or for other purposes. The

15   methods described herein allows for the systematic modification of viruses as necessary, such as the introduction of additional attenuating mutations into a recombinant virus in response to clinical studies.

As noted above, production of a sufficiently attenuated biologically derived HMPV or other viral mutant can be accomplished by several known methods. One such procedure involves

20   subjecting a partially attenuated virus to passage in cell culture at progressively lower, attenuating temperatures. For example, partially attenuated mutants are produced by passage in cell cultures at suboptimal temperatures. Thus, a cold-adapted (*ca*) mutant or other partially attenuated HMPV strain is adapted to efficient growth at a lower temperature by passage in culture. This selection of mutant HMPV during cold-passage substantially reduces any residual virulence in the derivative strains as

25   compared to the partially attenuated parent. Alternatively, specific mutations can be introduced into biologically derived HMPV by subjecting a partially attenuated parent virus to chemical mutagenesis, for example, to introduce ts mutations or, in the case of viruses which are already *ts*, additional ts mutations sufficient to confer increased attenuation and/or stability of the ts phenotype of the attenuated derivative. Means for the introduction of *ts* mutations into HMPV include replication of

30   the virus in the presence of a mutagen such as 5-fluorouridine according to generally known procedures. Other chemical mutagens can also be used. Attenuation can result from a *ts* mutation in almost any HMPV gene, although a particularly amenable target for this purpose has been found to be the polymerase (L) gene. The level of temperature sensitivity of replication in exemplary attenuated HMPV can be determined by comparing its replication at a permissive temperature with

35   that at several restrictive temperatures. The lowest temperature at which the replication of the virus is reduced 100-fold or more in comparison with its replication at the permissive temperature is termed

the "shutoff temperature." In experimental animals and humans, both the replication and virulence of HMPV correlate with the mutant's shutoff temperature.

From biologically and recombinantly derived HMPV and other nonsegmented negative stranded RNA viruses, a large "menu" of attenuating mutations is identifiable by the teachings herein,

5   each of which can be combined with any other mutations for adjusting the level of attenuation, immunogenicity and genetic stability in recombinant HMPV. In this context, many recombinant HMPV candidates will include one or more, and preferably two or more, mutations from a biologically derived HMPV or other heterologous viral mutant, for example, any one or combination of mutations identified in APV, HPIV, BPIV3, and/or RSV. Preferred recombinant HMPVs can

10   incorporate a plurality of mutations thus identified. Often, these mutations are stabilized against reversion in recombinant HMPV by multiple nucleotide substitutions in a codon specifying each mutation.

Mutations compiled into a "menu" as described above are introduced as desired, singly or in combination, to adjust recombinant HMPV to an appropriate level of attenuation, immunogenicity,

15   genetic resistance to reversion from an attenuated phenotype, etc. In accordance with the foregoing description, the ability to produce infectious recombinant HMPV from cDNA permits introduction of specific engineered changes within the recombinant HMPV. In particular, infectious, recombinant HMPVs can be employed for further identification of specific mutations in biologically derived, attenuated HMPV strains, for example mutations that specify *ts*, *ca*, *att* and other phenotypes.

20   Desired mutations identified by this and other methods are introduced into recombinant HMPV candidate strains. The capability of producing virus from cDNA allows for routine incorporation of these mutations, individually or in various selected combinations, into a full-length cDNA clone, where after the phenotypes of rescued recombinant viruses containing the introduced mutations can be readily determined.

25   By identifying and incorporating specific mutations associated with desired phenotypes, for example, a *ca* or *ts* phenotype, into infectious recombinant HMPV, additional site-specific modifications at, or within close proximity to, the identified mutation are identified. Whereas most attenuating mutations produced in biologically derived HMPVs are single nucleotide changes, other "site specific" mutations can also be incorporated by recombinant techniques into a recombinant

30   HMPV. As used herein, site-specific mutations include insertions, substitutions, deletions or rearrangements of from about 1 to about 3, up to about 5-15 or more altered nucleotides (for example, altered from a wild type HMPV sequence, from a sequence of a selected mutant HMPV strain, or from a parent recombinant HMPV clone subjected to mutagenesis). Such site-specific mutations can be incorporated at, or within the region of, a selected, biologically derived point mutation.

35   Alternatively, the mutations can be introduced in various other contexts within a recombinant HMPV clone, for example at or near a cis-acting regulatory sequence or nucleotide sequence encoding a protein active site, binding site, immunogenic epitope, etc.

Site-specific recombinant HMPV mutants typically retain a desired attenuating phenotype, but can additionally exhibit altered phenotypic characteristics unrelated to attenuation, for example, enhanced or broadened immunogenicity, and/or improved growth. Further examples of desired, site-specific mutants include recombinant HMPV mutants engineered to incorporate additional,

5   stabilizing nucleotide mutations in a codon specifying an attenuating point mutation. Where possible, two or more nucleotide substitutions are introduced at codons that specify attenuating amino acid changes in a parent mutant or recombinant HMPV clone, yielding a recombinant HMPV with greater genetic resistance to reversion from an attenuated phenotype. In other embodiments, site-specific nucleotide substitutions, additions, deletions or rearrangements are introduced upstream or

10   downstream, e. g., from about 1 to about 3, about 5-10 and up to 15 nucleotides or more 5' or 3', relative to a targeted nucleotide position, for example, to construct or ablate an existing cis-acting regulatory element.

In addition to single and multiple point mutations and site-specific mutations, changes to the recombinant HMPV include deletions, insertions, substitutions or rearrangements of one or more

15   genes or genome segments to increase, decrease, ablate or otherwise alter gene expression. Expression of one or more non-essential genes can be reduced or ablated by modifying the recombinant HMPV genome or antigenome, for example, to incorporate a mutation that alters the coding assignment of an initiation codon or mutations that introduce one or one or more stop codons. Alternatively, one or more non-essential genes or genome segments can be deleted in whole or in part

20   to render the corresponding proteins partially or entirely non-functional or to disrupt protein expression altogether. Exemplary recombinant HMPV within these aspects of the current disclosure are provided herein that exhibit partial or complete deletions of the G, SH and/or M2-2 ORFs, including an exemplary mutant that has both the G and SH genes deleted. These and other recombinants can be engineered and selected to possess highly desirable phenotypic characteristics

25   for development of immunogenic compositions. For example, these modifications can specify one or more desired phenotypic changes including (i) altered growth properties in cell culture, (ii) attenuation in the upper and/or lower respiratory tract of mammals, (iii) a change in viral plaque size, (iv) a change in cytopathic effect, (v) a change in immunogenicity, and (vi) a change in RNA replication and gene expression.

30   Thus a recombinant HMPV can incorporate one or more partial or complete gene deletions, knock out mutations, or mutations that simply reduce or increase expression of an HMPV gene. This can be achieved, for example, by introducing a frame shift mutation or termination codon within a selected coding sequence, altering translational start sites, changing the position of a gene or introducing an upstream start codon to alter its rate of expression, changing GS and/or GE

35   transcription signals to alter phenotype, or modifying an RNA editing site (for example, growth, temperature restrictions on transcription, etc.). In several examples, recombinant HMPVs are provided in which expression of one or more genes, for example, a SH, G, or M2-2 ORF, is ablated at

the translational or transcriptional level without deletion of the gene or of a segment thereof, by, for example, introducing multiple translational termination codons into a translational ORF, altering an initiation codon, or modifying an editing site. In one embodiment, expression of the M2-2 ORF is ablated by removal of both potential translational start codons, in combination with partial deletion of

5   the M2-2 ORF. These and other forms of knock-out virus will often exhibit reduced growth rates and small plaque sizes in tissue culture. Thus, additional novel types of attenuating mutations are provided herein which ablate or alter expression of a viral gene. In this context, knock-out virus phenotypes produced without deletion of a gene or genome segment can be alternatively produced by deletion mutagenesis, as described, to effectively preclude correcting mutations that may restore

10   synthesis of a target protein. Other gene knock-outs can be made using alternate designs and methods that are well known in the art (as described, for example, in (Kretschmer *et al.*, Virology 216:309-316, 1996; Radecke *et al.*, Virology 217:418-421, 1996; Kato *et al.*, EMBO J. 16:578-587, 1987; and Schneider *et al.*, Virology 277:314-322, 1996).

     Nucleotide modifications that may be introduced into recombinant HMPV constructs can

15   alter small numbers of bases (for example, from 15-30 bases, up to 35-50 bases or more), large blocks of nucleotides (for example, about 50-100, about 100-300, about 300-500, about 500-1,000 bases), or nearly complete or complete genes (for example, about 1,000-1,500 nucleotides, about 1,500-2,500 nucleotides, about 2,500-5,000, nucleotides, about 5,000-6,0000 nucleotides or more) in the vector genome or antigenome or heterologous, donor gene or genome segment, depending upon the nature

20   of the change (that is, a small number of bases may be changed to insert or ablate an immunogenic epitope or change a small genome segment, whereas large blocks of bases are involved when genes or large genome segments are added, substituted, deleted or rearranged).

     In related aspects, the current disclosure provides for supplementation of mutations adopted into a recombinant HMPV clone from biologically derived HMPV, for example, *ca* and *ts* mutations,

25   with additional types of mutations involving the same or different genes in a further modified recombinant HMPV. Each of the HMPV genes can be selectively altered in terms of expression levels, or can be added, deleted, substituted or rearranged, in whole or in part, alone or in combination with other desired modifications, to yield a recombinant HMPV exhibiting novel characteristics. Thus, in addition to or in combination with attenuating mutations adopted from

30   biologically derived HMPV and/or non-HMPV mutants, the current disclosure also provides a range of additional methods for attenuating or otherwise modifying the phenotype of a recombinant HMPV based on recombinant engineering of infectious HMPV clones. A variety of alterations can be produced in an isolated polynucleotide sequence encoding a targeted gene or genome segment, including a donor or recipient gene or genome segment in a recombinant HMPV genome or

35   antigenome for incorporation into partially attenuated, infectious clones. More specifically, to achieve desired structural and phenotypic changes in recombinant HMPV, the current disclosure allows for introduction of modifications which delete, substitute, introduce, or rearrange a selected

nucleotide or nucleotide sequence from a parent genome or antigenome, as well as mutations which delete, substitute, introduce or rearrange whole genes or genome segments, within a recombinant HMPV.

Thus provided are modifications in recombinant HMPV which simply alter or ablate
5   expression of a selected gene, for example, by introducing a termination codon within a selected HMPV coding sequence or altering its translational start site or RNA editing site, changing the position of a HMPV gene relative to an operably linked promoter, introducing an upstream start codon to alter rates of expression, modifying (for example, by changing position, altering an existing sequence, or substituting an existing sequence with a heterologous sequence) GS and/or GE
10   transcription signals to alter phenotype (for example, growth, temperature restrictions on transcription, etc.), and various other deletions, substitutions, additions and rearrangements that specify quantitative or qualitative changes in viral replication, transcription of selected genes, or translation of selected proteins. In this context, any HMPV gene or genome segment which is not essential for growth can be ablated or otherwise modified in a recombinant HMPV to yield desired
15   effects on virulence, pathogenesis, immunogenicity and other phenotypic characters. In addition to coding sequences, noncoding, leader, trailer and intergenic regions can be similarly deleted, substituted or modified and their phenotypic effects readily analyzed, for example, by the use of minireplicons, and the recombinant HMPV described herein.

In addition to these changes, the order of genes in a recombinant HMPV construct can be
20   changed, a HMPV genome promoter replaced with its antigenome counterpart or vice versa, portions of genes removed or substituted, and even entire genes deleted. Different or additional modifications in the sequence can be made to facilitate manipulations, such as the insertion of unique restriction sites in various intergenic regions or elsewhere. Nontranslated gene sequences can be removed to increase capacity for inserting foreign sequences.
25   Other mutations for incorporation into recombinant HMPV constructs of the current disclosure include mutations directed toward cis-acting signals, which can be readily identified, for example, by mutational analysis of HMPV minigenomes. For example, insertional and deletional analysis of the leader, trailer and/or flanking sequences identifies viral promoters and transcription signals and provides a series of mutations associated with varying degrees of reduction of RNA
30   replication or transcription. Saturation mutagenesis (whereby each position in turn is modified to each of the nucleotide alternatives) of these cis-acting signals also can be employed to identify many mutations that affect RNA replication or transcription. Any of these mutations can be inserted into a chimeric HMPV antigenome or genome as described herein. Evaluation and manipulation of trans-acting proteins and cis-acting RNA sequences using the complete antigenome cDNA is assisted by
35   the use of HMPV minigenomes as described in the above-incorporated references.

Additional mutations within recombinant HMPVs can also include replacement of the 3' end of genome with its counterpart from antigenome or vice versa, which is associated with changes in

RNA replication and transcription. In one exemplary embodiment, the level of expression of specific HMPV proteins, such as the protective HN and/or F antigens, can be increased by substituting the natural sequences with ones which have been made synthetically and designed to be consistent with efficient translation. In this context, it has been shown that codon usage can be a major factor in the

5   level of translation of mammalian viral proteins (Haas *et al.*, Current Biol. 6:315-324, 1996). Optimization by recombinant methods of the codon usage of the mRNAs encoding one or more immunogenic glycoproteins of recombinant HMPV will provide improved expression for these genes.

         In another exemplary embodiment, a sequence surrounding a translational start site

10   (preferably including a nucleotide in the -3 position relative to the AUG start site) of a selected HMPV gene or donor gene incorporated in an HMPV vector is modified, alone or in combination with introduction of an upstream start codon, to modulate gene expression by specifying up- or down-regulation of translation. Alternatively, or in combination with other recombinant modifications disclosed herein, gene expression of a recombinant HMPV can be modulated by altering a

15   transcriptional GS or GE signal of any selected genes of the virus. In alternative embodiments, levels of gene expression in a recombinant HMPV candidate are modified at the level of transcription. In one aspect, the position of a selected gene in the HMPV gene map can be changed to a more promoter-proximal or promoter-distal position, whereby the gene will be expressed more or less efficiently, respectively. According to this aspect, modulation of expression for specific genes can be

20   achieved yielding reductions or increases of gene expression from two-fold, more typically four-fold, up to ten-fold or more compared to wild type levels often attended by a commensurate decrease in expression levels for reciprocally, positionally substituted genes. These and other transpositioning changes yield novel recombinant HMPVs having attenuated phenotypes, for example due to decreased expression of selected viral proteins involved in RNA replication, or having other desirable

25   properties such as increased antigen expression.

         In the latter context, any one or combination of antigenic glycoproteins, prospectively including F, G, and SH, or antigenic determinants thereof, can be produced at elevated or decreased levels (or with otherwise enhanced immunogenic activity) by, for example, changing the promoter-relative position of the subject gene or genome segment. For example, the wild type gene order of

30   the SH, G and F ORFs of HMPV are located at gene positions 6, 7 and 4, respectively. Recombinantly altering the promoter proximity of one or more of these genes will modulated its expression, and/or expression of normally "upstream" and "downstream" genes. The change in gene expression due to the relocation of genes or to the addition of a second copy can have other desirable effects, such as increased or decreased growth *in vitro* or *in vivo*. In exemplary embodiments,

35   placement of putative protective antigen genes such as SH, G, and F more proximal to the promoter, either singly, as a pair, or as a triplet, will result in more efficient expression. As described herein, exemplary embodiments of "promoter-shifted" HMPV are provided wherein the position of the SH-G

gene pair was altered, for example by moving the pair from its wild type gene order position
following the M2 gene to a position preceding the F gene. This resulted in a gene order in that region
of the genome that mimics that of RSV. In another exemplary embodiment, the SH-G pair was
moved to be upstream of the F gene, and a second copy of the pair was inserted, resulting in multiple

5   copies of the SH and G prospective antigens. In yet additional embodiments, the prospective
antigens comprising one or more of the G and F proteins are modified in their expression by
promoter-relative shifting of these genes (having wild type gene order positions at the 7th and 4th
gene positions, respectively in the HMPV genome map) to more promoter-proximal positions.

        In other embodiments, recombinant HMPVs useful in immunogenic compositions can be

10   conveniently modified to accommodate antigenic drift in circulating virus. In this context, the
Examples below provide the complete sequence for an HMPV genome, and also provide a detailed
comparison of complete genome sequences for HMPV isolates representing two distinct antigenic
subgroups of HMPV, that are generally comparable in sequence divergence to the antigenic
subgroups of HRSV. Identification and characterization of these distinct antigenic subgroups,

15   including detailed mapping of their prospective antigenic glycoprotein gene structure, provides for
construction of novel recombinant HMPVs for use in immunogenic compositions to elicit an immune
response against one or more HMPV strains, including in bivalent immunogenic composition to
immunize against multiple HMPV strains and/or accommodate antigenic drift. Typically the
recombinant HMPV will have a modification in one or more of the SH, G, and/or F glycoproteins.

20   For example, an entire SH, G, and/or F gene, or a genome segment encoding a particular antigenic
determinant (for example, immunogenic region or epitopes) thereof), from one HMPV strain or group
can be incorporated into a recombinant HMPV genome or antigenome cDNA by replacement of a
corresponding region in a recipient clone of a different HMPV strain or group, or by adding one or
more copies of the gene, such that multiple antigenic forms are represented in the recombinant virus.

25   Progeny virus produced from the modified recombinant HMPV can then be used in immunization
protocols against multiple, and emerging, HMPV strains.

        In certain aspects of the current disclosure, replacement of a HMPV coding sequence or
non-coding sequence (for example, a promoter, GE, GS, intergenic or other cis-acting element) with a
heterologous (for example, non-HMPV) counterpart yields chimeric HMPV having a variety of

30   possible attenuating and other phenotypic effects. For example, host range and other desired effects
can be engineered by importing an APV or other non-HMPV virus (for example, RSV, SV5, SV41,
NDV, PIV) protein, protein domain, gene or genome segment into a recombinant HMPV
"background" genome or antigenome, wherein the heterologous or "donor" gene or genome segment
does not function efficiently in a human cell, for example, from incompatibility of the heterologous

35   sequence or protein with a biologically interactive HMPV sequence or protein (that is, a sequence or
protein that ordinarily cooperates with the substituted sequence or protein for viral transcription,
translation, assembly, etc.) or, more typically in a host range restriction, with a cellular protein or

some other aspect of the cellular milieu which is different between the permissive and less permissive host. In exemplary embodiments, APV, pneumovirus of mice, or RSV sequences are selected for introduction into HMPV based on aspects of HMPV structure and function described herein.

Methods are provided herein for attenuating recombinant HMPV candidates based on the
5   construction of chimeras between HMPV and a non-human pneumovirus or other negative stranded RNA virus, for example APV, RSV, HPIV, MPIV1 (Sendai virus), BPIV3, SV5, SV41, and/or NDV (for example, as disclosed in U.S. Patent Application Serial No. 09/586,479, filed June 1, 2000 by Schmidt et al. (corresponding to PCT Publication WO 01/04320); Schmidt et al., J. Virol. 74:8922-9, 2000). In exemplary embodiments, the recombinant HMPV genome or antigenome is combined with
10   a heterologous gene or genome segment, such as an N, P, M, or L, ORF derived from a non-HMPV paramyxovirus.

Paramyxoviruses are enveloped RNA viruses that include two subfamilies (Paramyxovirinae and Pneumovirinae). Subfamily Paramyxovirinae has five different genera, including Respirovirus (containing parainfluenza viruses 1 and 3), Rubulavirus (containing
15   parainfluenza viruses 2 and 4 and mumps), and Morbillivirus (containing measles and canine distemper viruses). Subfamily Pneumovirinae has two genera, namely Pneumovirus (containing RSV) and Metapneumovirus (containing HMPV). Paramyxoviruses encoded 6-11 proteins. Proteins common to all paramyxoviruses include the nucleocapsid N protein that associates tightly with the RNA genome to form the nucleocapsid, the phosphoprotein P and large L polymerase protein that
20   also associate with the nucleocapsid, the matrix M protein that associates with the inner face of the viral envelope, the transmembrane fusion F glycoprotein that mediates penetration, and the attachment protein that, depending on the virus, is called glycoprotein G, or the hemagglutinin HA or the hemagglutinin-neuraminidase HN. Other proteins are found in some viruses but not others, such as the nonstructural NS1 and NS2 proteins of RSV, the M2-1 and M2-2 proteins found in RSV and,
25   as disclosed here, in HMPV, the SH protein that is found in mumps, simian virus 5, RSV and HMPV, and the C, D and V proteins found in certain parainfluenza viruses. The sequences of non-HMPV HN, M, N, P, L, F, G, and SH proteins, and nucleic acids encoding these proteins are known in the art (see, Lamb, RA and Kolakofsky, D. pp 1305-1340 in Fields Virology, 4th addition, Knipe and Howley (eds) Lippincott Williams and Wilkins, Philadelphia, 2001, Chanock, Murphy and Collins,
30   pp 1341-1379, ibid, Collins, Chanock and Murphy, pp 1443-1485, ibid.).

Chimeric HMPV are therefore provided herein that include a partial or complete "background" HMPV genome or antigenome derived from or patterned after HMPV combined with one or more heterologous genes or genome segments of a non- HMPV virus to form the chimeric HMPV genome or antigenome. In one embodiment, chimeric HMPV of this type incorporate a
35   partial or complete HMPV background genome or antigenome combined with one or more heterologous genes or genome segments. The partial or complete background genome or antigenome typically acts as a recipient backbone into which the heterologous genes or genome segments of the

counterpart, non- HMPV virus are incorporated. Heterologous genes or genome segments from the
counterpart virus represent "donor" genes or polynucleotides that are combined with, or substituted
within, the background genome or antigenome to yield a chimeric HMPV that exhibits novel
phenotypic characteristics compared to one or both of the contributing viruses. For example,

5   addition or substitution of heterologous genes or genome segments within a selected recipient HMPV
strain may result in an increase or decrease in attenuation, growth changes, altered immunogenicity,
or other desired phenotypic changes as compared with a corresponding phenotypes of the unmodified
recipient and/or donor (see, for example, U.S. Patent Application Serial No. 09/586,479, filed June 1,
2000 by Schmidt et al.; Schmidt et al., J. Virol. 74:8922-9, 2000).

10   Genes and genome segments that can be selected for use as heterologous substitutions or
additions within chimeric HMPV include genes or genome segments encoding a HMPV N, P, M, F,
M2, SH, G and/or L proteins or portions thereof. In addition, genes and genome segments encoding
proteins found in other viruses, (for example, an SH protein as found in mumps, RSV, and SV5
viruses), may be incorporated within additional chimeric HMPV recombinants of the current

15   disclosure. Regulatory regions from heterologous viruses, for example the extragenic 3' leader or 5'
trailer regions, and GS, GE, intergenic regions, or 3' or 5' non-coding regions, are also useful as
substitutions or additions within recombinant HMPV. In exemplary aspects, chimeric HMPV
bearing one or more non-HMPV genes or genome segments exhibit a high degree of host range
restriction, for example, in the respiratory tract of mammalian models of human HMPV infection

20   such as hamsters and non-human primates. In more detailed embodiments HMPV is attenuated by
the addition or substitution of one or more APV or RSV genes or genome segments selected from N,
P, M, F, M2, SH, G and/or L genes and genome segments to a partial or complete HMPV background
genome or antigenome.

In one embodiment, the degree of host range restriction exhibited by chimeric HMPV for

25   use within immunogenic compositions of the current disclosure is comparable to the degree of host
range restriction exhibited by the respective non-HMPV "donor" strain. For example, the restriction
should have a true host range phenotype, that is, it should be specific to the host in question and
should not restrict replication in vitro in a suitable cell line. In addition, chimeric HMPV bearing one
or more heterologous genes or genome segments elicit a desired immunogenic response in hosts

30   susceptible to HMPV infection. Thus, the current disclosure provides a new basis for attenuating a
live HMPV virus vector for developing immunogenic compositions against HMPV and other
pathogens based on host range effects.

In combination with the host range phenotypic effects provided in the chimeric HMPV, it is
often desirable to adjust the attenuation phenotype by introducing additional mutations that increase

35   or decrease attenuation of the chimeric virus. Thus, in additional embodiments, attenuated, chimeric
HMPV are produced in which the chimeric genome or antigenome is further modified by introducing
one or more attenuating mutations specifying an attenuating phenotype in the resultant virus or

subviral particle. These can include mutations generated de novo and tested for attenuating effects according to a rational design mutagenesis strategy. Alternatively, the attenuating mutations may be identified in existing biologically derived mutant HMPV or non-HMPVs and thereafter incorporated into a chimeric HMPV of the current disclosure. Exemplary mutations specify lesions in RNA

5   regulatory sequences or in encoded proteins.

In certain chimeric HMPV, attenuation marked by replication in the lower and/or upper respiratory tract in an accepted animal model that is reasonably correlated with HMPV replication and immunogenic activity in humans (for example, hamsters, rhesus monkeys or chimpanzees), is reduced by at least about two-fold, more often about 5-fold, 10-fold, or 20-fold, and preferably 50-

10   100-fold and up to 1,000-fold or greater overall (for example, as measured between 3-8 days following infection) compared to growth of the corresponding wild type or mutant parental strains.

Within the methods disclosed herein, additional genes or genome segments can be inserted into or proximate to a recombinant or chimeric HMPV genome or antigenome. For example, various supernumerary heterologous genes or genome segments can be inserted at any of a variety of sites

15   within the recombinant genome or antigenome, for example at a position 3' to N, between the N/P, P/M, M/F, F/M2, M2/SH, SH/G, and/or G/L genes, or at another non-coding region of the HMPV vector genome or antigenome (see FIG. 1). The inserted genes can be under common control with recipient genes, or can be under the control of an independent set of transcription signals. Genes of interest in this context include genes encoding cytokines, for example, an interleukin (IL-2 through

20   IL-18, for example, interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 18 (IL-18), tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), or granulocyte-macrophage colony stimulating factor (GM-CSF) (see, for example, U.S. Application No. 09/614,285, filed July 12, 2000 and priority U.S. Provisional Application Serial No. 60/143,425 filed July 13, 1999). Coexpression of these additional proteins provides the ability to modify and

25   improve immune responses against recombinant HMPV quantitatively and/or qualitatively.

In additional embodiments, insertion of heterologous nucleotide sequences into recombinant HMPV candidates are employed separately to modulate the level of attenuation of candidate recombinants, for example, for the upper respiratory tract. Thus, it is possible to insert nucleotide sequences into a recombinant HMPV that both direct the expression of a foreign protein and that

30   attenuate the virus in an animal host, or to use nucleotide insertions separately to attenuate candidate viruses. To define some of the rules that govern the effect of gene insertion on attenuation, gene units of varying lengths are inserted into a wild type HMPV backbone and the effects of gene unit length on attenuation examined. Novel gene unit insertions are contemplated in this regard that do not contain a significant ORF, permitting identification of the effect of gene unit length independently of

35   an effect of the expressed protein of that gene. These heterologous sequences may be inserted as an extra gene unit of various sizes, for example, from about 150 or more nts in length up to 3,000 nts or more in length. Gene unit (GU) insertions of about 1,000 or 2,000 nts in length will often

substantially attenuate rHMPV candidates for the upper respiratory tract of mammalian subjects. In
addition, gene unit insertions can have the dual effect of both attenuating a candidate virus and
inducing an immunogenic response against a second virus. Alternately, gene extensions in the 3'-
noncoding region (NCR) of a HMPV gene, which cannot express additional proteins, can also be

5   attenuating in and of themselves. Within these methods of the current disclosure, gene insertion
length is a determinant of attenuation (see, for example, U.S. Patent Application No. 10/302,547,
filed November 21, 2002 and priority U.S. Provisional Application No. 60/331,961, filed November
21, 2001; and U.S. Provisional Application No. 60/412,053, filed September 18, 2002).

        Deletions, insertions, substitutions and other mutations involving changes of whole viral
10   genes or genome segments within rHMPV yield highly stable candidates, which are particularly
important in the case of immunosuppressed individuals. Many of these changes will result in
attenuation of resultant strains, whereas others will specify different types of desired phenotypic
changes. For example, accessory (that is, not essential for *in vitro* growth) genes are excellent
candidates to encode proteins that specifically interfere with host immunity (see, for example, Kato *et*
15   *al.*, EMBO. J. 16:578-87, 1997). Ablation of such genes in candidate viruses can reduce virulence
and pathogenesis and/or improve immunogenicity.

        In more detailed embodiments, chimeric HMPVs are constructed using a HMPV "vector"
genome or antigenome that is recombinantly modified to incorporate one or more antigenic
determinants of a heterologous pathogen. The vector genome or antigenome includes a partial or
20   complete HMPV genome or antigenome, which may itself incorporate nucleotide modifications such
as attenuating mutations. The vector genome or antigenome is modified to form a chimeric structure
through incorporation of a heterologous gene or genome segment. More specifically, chimeric
HMPVs of the current disclosure are constructed through a cDNA-based virus recovery system that
yields recombinant viruses that incorporate a partial or complete vector or "background" HMPV
25   genome or antigenome combined with one or more "donor" nucleotide sequences encoding the
heterologous antigenic determinants. In exemplary embodiments a HMPV vector genome or
antigenome is modified to incorporate one or more genes or genome segments that encode antigenic
determinants of one or more heterologous negative stranded RNA viruses (for example, another
HMPV, RSV, PIV, or measles virus). Thus constructed, chimeric HMPVs can elicit, for example, an
30   immune response against a specific HMPV or against a non-HMPV pathogen. Alternatively,
compositions and methods are provided employing a HMPV-based chimeric virus to elicit a
polyspecific immune response against multiple HPIVs, against one or more HMPVs and a non-PIV
pathogen such as RSV, PIV, or measles virus.

        A chimeric HMPV in this context can incorporate a partial or complete human HMPV
35   incorporating one or more heterologous polynucleotides encoding one or more antigenic determinants
of the heterologous pathogen. These heterologous polynucleotides can be added to or substituted
within the HMPV vector genome or antigenome to yield the chimeric HMPV recombinant. The

chimeric HMPV virus thus acquires the ability to elicit an immune response in a selected host against the heterologous pathogen. In addition, the chimeric virus can exhibit other novel phenotypic characteristics compared to one or both of the vector HMPV and heterologous pathogens. In addition to providing novel immunogenic characteristics, the addition or substitution of heterologous genes or

5   genome segments within the vector HMPV strain may confer an increase or decrease in attenuation, growth changes, or other desired phenotypic changes as compared with the corresponding phenotypes of the unmodified vector and donor viruses.

Heterologous genes or genome segments of a different HMPV or non-HMPV virus can be added as a supernumerary genomic element to a partial or complete genome or antigenome of a

10   rHMPV. Alternatively, one or more heterologous genes or genome segments may be substituted into the rHMPV at a position corresponding to a wild type gene order position of a counterpart genes or genome segments that is deleted within the HMPV vector genome or antigenome. In yet additional embodiments, the heterologous gene or genome segment is added or substituted at a position that is more promoter-proximal or promoter-distal compared to a wild type gene order position of the

15   counterpart gene or genome segment within the vector genome or antigenome to enhance or reduce, respectively, expression of the heterologous gene or genome segment. Additional methods and compositions that are useful for engineering chimeric HMPV employ certain known techniques, for example, as disclosed for PIV viruses (Durbin et al., Virology 235:323-332, 1997; Skiadopoulos et al., J. Virol. 72:1762-1768, 1998; Tao et al., J Virol 72:2955-2961, 1998; Skiadopoulos et al., J.

20   Virol. 73:1374-1381, 1999; Skiadopoulos et al., Vaccine 18:503-510, 1999; Tao et al., Vaccine 17:1100-1108, 1999; Tao et al., Vaccine 18:1359-1366, 2000; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Patent Application Serial No. 09/458,813, filed December 10, 1999; U.S. Patent Application Serial No. 09/459,062, filed December 10, 1999; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO

25   98/53078), and U.S. Provisional Application No. 60/059,385, filed September 19, 1997).

Chimeric HMPV can also be constructed that express a chimeric protein, for example an immunogenic glycoprotein having a cytoplasmic tail and/or transmembrane domain specific to a HMPV vector fused to a heterologous ectodomain (the ectodomain being that part of a transmembrane surface protein that extends into the luminal or extracellular space) of a different

30   HMPV or non-HMPV pathogen to provide a fusion protein that elicits an immune response against the heterologous pathogen. For example, a heterologous genome segment encoding a glycoprotein ectodomain from a RSV F, SH, M2, or G glycoprotein can be joined with a genome segment encoding the corresponding HMPV glycoprotein cytoplasmic and transmembrane domains to form a chimeric glycoprotein that elicits an immune response against RSV.

35   Briefly, HMPV expressing a chimeric glycoprotein includes a major nucleocapsid protein, a nucleocapsid phosphoprotein, a large polymerase protein, and a HMPV vector genome or antigenome that is modified to encode a chimeric glycoprotein. The chimeric glycoprotein incorporates one or

more heterologous antigenic domains, fragments, or epitopes of a second, antigenically distinct HMPV or non-HMPV pathogen. In one embodiment, this is achieved by substitution within the HMPV vector genome or antigenome of one or more heterologous genome segments of the second virus that encodes one or more antigenic domains, fragments, or epitopes, whereby the recombinant

5   genome or antigenome encodes the chimeric glycoprotein that is antigenically distinct from the parent, vector virus. In several examples, the heterologous genome segment or segments can encode a glycoprotein ectodomain or immunogenic portion or epitope thereof. The heterologous genome segment can also include other portions of the heterologous or "donor" glycoprotein, for example both an ectodomain and transmembrane region that are substituted for counterpart glycoprotein ecto-

10   and transmembrane domains in the vector genome or antigenome.

As used herein, the term "gene" generally refers to a portion of a subject genome, for example, a HMPV genome, encoding an mRNA and typically begins at the upstream end with a GS signal and ends at the downstream end with the GE signal. The term gene is also interchangeable with the term "translational open reading frame," or "ORF," particularly in the case where a protein,

15   such as the HPIV1 or HPIV3 C protein, is expressed from an additional ORF rather than from a unique mRNA. The viral genome of all mononegaviruses also contains extragenic leader and trailer regions, possessing part of the promoters required for viral replication and transcription, as well as non-coding and intergenic regions. Transcription initiates at the 3' end and proceeds by a sequential stop-start mechanism that is guided by short conserved motifs found at the gene boundaries. The

20   upstream end of each gene contains a GS signal, that directs initiation of its respective mRNA. The downstream terminus of each gene contains a GE motif that directs polyadenylation and termination. The current disclosure provides operative identification of transcription signals and insertion sites, and provides the identification of the GS, GE and potential insertion sites within the genome.

To construct chimeric HMPVs, one or more HMPV genes or genome segments can be

25   deleted, inserted or substituted in whole or in part. This means that partial or complete deletions, insertions and substitutions may include ORFs and/or cis-acting regulatory sequences of any one or more of the HMPV genes or genome segments. By "genome segment" is meant any length of continuous nucleotides from the HMPV genome, which might be part of an ORF, a gene, or an extragenic region, or a combination thereof. In several examples, at least about 10, at least about 20,

30   at least about 30, at least about 50, or at least about 100 continuous nucleotides are deleted, inserted or substituted. When a subject genome segment encodes an antigenic determinant, the genome segment encodes at least one immunogenic epitope capable of eliciting a humoral or cell mediated immune response in a mammalian host. The genome segment can also encode an immunogenic fragment or protein domain. For example, the donor genome segment can encode multiple

35   immunogenic domains or epitopes, including recombinantly synthesized sequences that comprise multiple, repeating or different, immunogenic domains or epitopes.

Chimeric HMPV can be engineered to express one or more major antigenic determinants of
a wide range of non-HMPV pathogens. The methods disclosed herein are generally adaptable for
incorporation of antigenic determinants from, for example, subgroup A and subgroup B RSVs,
HMPV, measles virus, mumps virus, human papilloma viruses, type 1 and type 2 human

5   immunodeficiency viruses, herpes simplex viruses, cytomegalovirus, rabies virus, Epstein Barr virus,
filoviruses, bunyaviruses, flaviviruses, alphaviruses and influenza viruses, among other pathogens.
Pathogens that can be targeted for development of immunogenic compositions include viral and
bacterial pathogens, as well as protozoans and multicellular pathogens. Useful antigenic
determinants from many important human pathogens in this context are known or readily identified

10   for incorporation within chimeric HMPV. Thus, major antigens have been identified for the
foregoing exemplary pathogens, including the measles virus HA and F proteins; the F, G, SH and M2
proteins of RSV, mumps virus HN and F proteins, human papilloma virus L1 protein, type 1 or type 2
human immunodeficiency virus gp160 protein, herpes simplex virus and cytomegalovirus gB, gC,
gD, gE, gG, gH, gI, gJ, gK, gL, and gM proteins, rabies virus G protein, Epstein Barr Virus gp350

15   protein; filovirus G protein, bunyavirus G protein, flavivirus E and NS1 proteins, and alphavirus E
protein. These major antigens, as well as other antigens known in the art for the enumerated
pathogens and others, are well characterized to the extent that many of their antigenic determinants,
including the full length proteins and their constituent antigenic domains, fragments and epitopes, are
identified, mapped and characterized for their respective immunogenic activities.

20   Among the numerous, exemplary mapping studies that identify and characterize major
antigens of diverse pathogens for use in the methods a chimeric HMPV disclosed herein are epitope
mapping studies directed, for example, to immunogenic glycoproteins of PIV. Exemplifying the
subject methods and tools, van Wyke Coelingh et al. (J. Virol. 63:375-382, 1989) described twenty-
six monoclonal antibodies (MAbs) (14 neutralizing and 12 nonneutralizing) that were used to

25   examine the antigenic structure, biological properties, and natural variation of the fusion (F)
glycoprotein of HPIV3. Analysis of laboratory-selected antigenic variants and of PIV3 clinical
isolates indicated that panel of MAbs recognizes at least 20 epitopes, 14 of which participate in
neutralization. Competitive binding assays confirmed that the 14 neutralization epitopes are
organized into three nonoverlapping principal antigenic regions (A, B, and C) and one bridge site

30   (AB), and that the 6 nonneutralization epitopes form four sites (D, E, F, and G). Most of the
neutralizing MAbs were involved in nonreciprocal competitive binding reactions, suggesting that
they induce conformational changes in other neutralization epitopes. These and related methods will
serve to readily determine candidate antigenic determinants among heterologous viruses for
expression by HMPV vectors of the current disclosure.

35   Other antigenic determinants for use within the current disclosure have been identified and
characterized for RSV. For example, Beeler et al., J. Virol. 63:2941-2950, 1989, employed eighteen
neutralizing monoclonal antibodies (MAbs) specific for the fusion glycoprotein of the A2 strain of

RSV to construct a detailed topological and operational map of epitopes involved in RSV neutralization and fusion. Competitive binding assays identified three nonoverlapping antigenic regions (A, B, and C) and one bridge site (AB). Thirteen MAb-resistant mutants (MARMs) were selected, and the neutralization patterns of the MAbs with either MARMs or RSV clinical strains

5   identified a minimum of 16 epitopes. MARMs selected with antibodies to six of the site A and AB epitopes displayed a small-plaque phenotype, which is consistent with an alteration in a biologically active region of the F molecule. Analysis of MARMs also indicated that these neutralization epitopes occupy topographically distinct but conformationally interdependent regions with unique biological and immunological properties. Antigenic variation in F epitopes was then examined by using 23

10   clinical isolates (18 subgroup A and 5 subgroup B) in cross-neutralization assays with the 18 anti-F MAbs. This analysis identified constant, variable, and hypervariable regions on the molecule and indicated that antigenic variation in the neutralization epitopes of the RSV F glycoprotein is the result of a noncumulative genetic heterogeneity. Of the 16 epitopes, 8 were conserved on all or all but 1 of 23 subgroup A or subgroup B clinical isolates. These antigenic determinants, including the full

15   length proteins and their constituent antigenic domains, fragments and epitopes, all represent useful candidates for integration within chimeric HMPV of the current disclosure to elicit novel immune responses as described above. (See also, Anderson *et al.*, J. Infect. Dis. 151:626-633, 1985; Coelingh *et al.*, J. Virol. 63:375-382, 1989; Fenner *et al.*, Scand. J. Immunol. 24:335-340, 1986; Fernie *et al.*, Proc. Soc. Exp. Biol. Med. 171:266-271, 1982; Sato *et al.*, J. Gen. Virol. 66:1397-1409, 1985; Walsh

20   *et al.*, J. Gen. Virol. 67:505-513, 1986, and Olmsted *et al.*, J. Virol. 63:411-420, 1989).

To express antigenic determinants of heterologous HMPVs and non-HMPV pathogens, numerous methods and constructs are provided herein. In certain detailed embodiments, a transcription unit comprising an ORF of a gene encoding an antigenic protein (for example, an RSV F or G gene) is added to a HMPV vector genome or antigenome at various positions, yielding

25   exemplary chimeric "vector" candidates. In exemplary embodiments, chimeric HMPVs are engineered that incorporate heterologous nucleotide sequences encoding protective antigens from RSV to produce infectious, attenuated candidates (see U.S. Patent Application No. 08/720,132, filed September 27, 1996, corresponding to International Publication WO 97/12032 published April 04/03/97, and U.S. Provisional Patent Application No. 60/007,083, filed September 27, 1995; U.S.

30   Provisional Patent Application No. 60/021,773, filed July 15, 1996; U.S. Provisional Patent Application No. 60/046,141, filed May 9, 1997; U.S. Provisional Patent Application No. 60/047,634, filed May 23, 1997; U.S. Patent Application No. 08/892,403, filed July 15, 1997 corresponding to International Publication No. WO 98/02530 published on January 22, 1998; U.S. Patent Application No. 09/291,894, filed on April 13, 1999 corresponding to International Publication No. WO 00/61611

35   published October 19, 2000, and priority U.S. Provisional Patent Application Serial No. 60/129,006, filed on April 13, 1999; U.S. Patent Application No. 09/602,212, filed June 23, 2000 and corresponding International Publication No. WO 01/04335 published on January 18, 2001, and

priority U.S. Provisional Patent Application Nos. 60/129,006, filed April 13, 1999, 60/143,097, filed July 9, 1999, and 60/143,132, filed July 9, 1999; International Publication No. WO 00/61737 published on October 19, 2000; Collins *et al.*, Proc Nat. Acad. Sci. U.S.A. 92:11563-11567, 1995; Bukreyev *et al.*, J. Virol. 70:6634-41, 1996, Juhasz *et al.*, J. Virol. 71:5814-5819, 1997; Durbin *et al.*,

5 Virology 235:323-332, 1997; He *et al.* Virology 237:249-260, 1997; Baron *et al.* J. Virol. 71:1265-1271, 1997; Whitehead *et al.*, Virology 247:232-9, 1998a; Whitehead *et al.*, J. Virol. 72:4467-4471, 1998b; Jin *et al.* Virology 251:206-214, 1998; and Whitehead *et al.*, J. Virol. 73:3438-3442, 1999, and Bukreyev *et al.*, Proc. Nat. Acad. Sci. U.S.A. 96:2367-72, 1999). Other reports and discussion incorporated or set forth herein identify and characterize RSV antigenic determinants that are useful

10 within the current disclosure.

HMPV chimeras incorporating one or more RSV antigenic determinants, can include a HMPV vector genome or antigenome combined with a heterologous gene or genome segment encoding an antigenic RSV glycoprotein, protein domain (for example, a glycoprotein ectodomain) or one or more immunogenic epitopes. In one embodiment, one or more genes or genome segments

15 from RSV SH, M2, F, and/or G genes is/are combined with the HMPV vector genome or antigenome to form the chimeric HMPV candidate. Certain of these constructs will express chimeric proteins, for example fusion proteins having a cytoplasmic tail and/or transmembrane domain of HMPV fused to an ectodomain of a corresponding RSV glycoprotein to yield a novel attenuated virus that optionally elicits a multivalent immune response against both HMPV and RSV.

20 In certain embodiments, it is useful to administer immunogenic compositions comprising a rHMPV in a predetermined schedule with one or more additional immunogenic components, for example a second immunogenic composition against RSV or PIV administered before or after the anti-HMPV composition. RSV and HPIV3 cause significant illness within the first four months of life whereas most of the illness caused by HPIV1 and HPIV2 occur after six months of age (Chanock

25 *et al.*, in Parainfluenza Viruses, Knipe *et al.* (Eds.), pp. 1341-1379, Lippincott Williams & Wilkins, Philadelphia, 2001; Collins *et al.*, In Fields Virology, Vol. 1, pp. 1205-1243, Lippincott-Raven Publishers, Philadelphia, 1996; Reed *et al.*, J. Infect. Dis. 175:807-13, 1997). Accordingly, certain sequential immunization protocols involve administration of a immunogenic composition as described herein that elicits an immune response against HMPV before, simultaneous with (for

30 example, as a combined immunogenic composition), or subsequent to, administration of a second immunogenic composition directed toward another virus. In one embodiment, an immunogenic composition that elicits an immune response against HMPV, or against HMPV and RSV is administered one, two or more times early in life, with the first dose administered at or before one month of age, followed by an immunogenic composition against HPIV1 and/or HPIV2 at about four

35 and six months of age. Alternatively, it might be advantageous to administer immunogenic components against HMPV, RSV, and one or more PIVs at the same time, perhaps as a multi-component immunogenic composition.

Thus, combinatorial immunogenic compositions and coordinate immunization protocols are provided herein for multiple pathogenic agents, including HMPV, RSV, and one or more PIVs. In exemplary embodiments, these methods and formulations are temporally selected to target early immunization against HMPV, RSV and/or PIV3. One exemplary immunization sequence employs

5   one or more live attenuated immunogenic compositions against HMPV, RSV and/or PIV3 as early as one month of age (for example, at one and two months of age) followed by mono- or bivalent PIV1 and/or PIV2 immunogenic composition at four and six months of age. The methods disclosed herein can be used to administer multiple immunogenic compositions, including one or more chimeric HMPV candidates, coordinately, for example, simultaneously in a mixture or separately in a defined

10   temporal sequence (for example, in a daily or weekly sequence), wherein each virus preferably expresses, for example, a different heterologous protective antigen. Such a coordinate/sequential immunization strategy, which is able to induce secondary antibody responses to multiple viral respiratory pathogens, provides a highly powerful and extremely flexible immunization regimen that is driven by the need to immunize against multiple pathogens in early infancy.

15   As noted above, the current disclosure permits a wide range of alterations to be recombinantly produced within the HMPV genome or antigenome, yielding defined mutations that specify desired phenotypic changes. Defined mutations can be introduced by a variety of conventional techniques (for example, site-directed mutagenesis) into a cDNA copy of the genome or antigenome. The use of genomic or antigenomic cDNA subfragments to assemble a complete

20   genome or antigenome cDNA as described herein has the advantage that each region can be manipulated separately, where small cDNA constructs provide for better ease of manipulation than large cDNA constructs, and then readily assembled into a complete cDNA. Thus, the complete antigenome or genome cDNA, or a selected subfragment thereof, can be used as a template for oligonucleotide-directed mutagenesis. This can be through the intermediate of a single-stranded

25   phagemid form, such as using the MUTA-gen® kit of Bio-Rad Laboratories (Richmond, CA), or a method using the double-stranded plasmid directly as a template such as the Chameleon® mutagenesis kit of Strategene (La Jolla, CA), or by the PCR employing either an oligonucleotide primer or a template which contains the mutations of interest. A mutated subfragment can then be assembled into the complete antigenome or genome cDNA. A variety of other mutagenesis

30   techniques are known and can be routinely adapted for use in producing the mutations of interest in a HMPV antigenome or genome cDNA of the current disclosure.

Thus, in one illustrative embodiment mutations are introduced by using the MUTA-gene® phagemid *in vitro* mutagenesis kit available from Bio-Rad Laboratories. In brief, cDNA encoding a HMPV genome or antigenome is cloned into the plasmid pTZ18U, and used to transform CJ236 cells

35   (Life Technologies). Phagemid preparations are prepared as recommended by the manufacturer. Oligonucleotides are designed for mutagenesis by introduction of an altered nucleotide at the desired

position of the genome or antigenome. The plasmid containing the genetically altered genome or antigenome is then amplified.

Mutations can vary from single nucleotide changes to the introduction, deletion or replacement of large cDNA segments containing one or more genes or genome segments. Genome

5   segments can correspond to structural and/or functional domains, for example, cytoplasmic, transmembrane or ectodomains of proteins, active sites such as sites that mediate binding or other biochemical interactions with different proteins, epitopic sites, for example, sites that stimulate antibody binding and/or humoral or cell mediated immune responses, etc. Useful genome segments in this regard range from about 15-35 nucleotides in the case of genome segments encoding small

10   functional domains of proteins, for example, epitopic sites, to about 50, about 75, about 100, about 200-500, and about 500-1,500 or more nucleotides.

The ability to introduce defined mutations into infectious recombinant HMPV has many applications, including the manipulation of HMPV pathogenic and immunogenic mechanisms. For example, the functions of HMPV proteins, including the N, P, M, F, M2, SH, G and/or L proteins can

15   be manipulated by introducing mutations which ablate or reduce the level of protein expression, or which yield mutant protein. Various genome RNA structural features, such as promoters, intergenic regions, and transcription signals, can also be routinely manipulated within the methods and compositions of the current disclosure. The effects of trans-acting proteins and cis-acting RNA sequences can be readily determined, for example, using a complete antigenome cDNA in parallel

20   assays employing HMPV minigenomes (Dimock *et al.*, J. Virol. 67:2772-8, 1993 in its entirety), whose rescue-dependent status is useful in characterizing those mutants that may be too inhibitory to be recovered in replication-independent infectious virus.

Certain substitutions, insertions, deletions or rearrangements of genes or genome segments within recombinant HMPV (for example, substitutions of a genome segment encoding a selected

25   protein or protein region, for instance a cytoplasmic tail, transmembrane domain or ectodomain, an epitopic site or region, a binding site or region, an active site or region containing an active site, etc.) are made in structural or functional relation to an existing, "counterpart" gene or genome segment from the same or different HMPV or other source. Such modifications yield novel recombinants having desired phenotypic changes compared to wild type or parental HMPV or other viral strains.

30   For example, recombinants of this type may express a chimeric protein having a cytoplasmic tail and/or transmembrane domain of one HMPV, or of a non-HMPV virus such as PIV or RSV, fused to an ectodomain of a rHMPV of the current disclosure. Other exemplary recombinants of this type express duplicate protein regions, such as duplicate immunogenic regions.

As used herein, "counterpart" genes, genome segments, proteins or protein regions, are

35   typically from heterologous sources (for example, from different HMPV genes, or representing the same (that is, homologous or allelic) gene or genome segment in different HMPV types or strains). Typical counterparts selected in this context share gross structural features, for example, each

counterpart can encode a comparable protein or protein structural domain, such as a cytoplasmic

domain, transmembrane domain, ectodomain, binding site or region, epitopic site or region, etc.

Counterpart domains and their encoding genome segments embrace an assemblage of species having

a range of size and sequence variations defined by a common biological activity among the domain or

5   genome segment variants.

Counterpart genes and genome segments, as well as other polynucleotides disclosed herein

for producing recombinant HMPV, often share substantial sequence identity with a selected

polynucleotide "reference sequence," for example, with another selected counterpart sequence. As

used herein, a "reference sequence" is a defined sequence used as a basis for sequence comparison,

10   for example, a segment of a full-length cDNA or gene, or a complete cDNA or gene sequence.

Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides

in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) include

a sequence (that is, a portion of the complete polynucleotide sequence) that is similar between the

two polynucleotides, and (2) can further include a sequence that is divergent between the two

15   polynucleotides, sequence comparisons between two (or more) polynucleotides are typically

performed by comparing sequences of the two polynucleotides over a "comparison window" to

identify and compare local regions of sequence similarity. A "comparison window", as used herein,

refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide

sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein

20   the portion of the polynucleotide sequence in the comparison window may comprise additions or

deletions (that is, gaps) of 20 percent or less as compared to the reference sequence (which does not

comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of

sequences for aligning a comparison window may be conducted by the local homology algorithm of

Smith & Waterman, (Adv. Appl. Math. 2:482, 1981), by the homology alignment algorithm of

25   Needleman & Wunsch, (J. Mol. Biol. 48:443, 1970), by the search for similarity method of Pearson

& Lipman, (Proc. Natl. Acad. Sci. U.S.A. 85:2444, 1988), by computerized implementations of these

algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package

Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection, and the

best alignment (that is, resulting in the highest percentage of sequence similarity over the comparison

30   window) generated by the various methods is selected. The term "sequence identity" means that two

polynucleotide sequences are identical (that is, on a nucleotide-by-nucleotide basis) over the window

of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally

aligned sequences over the window of comparison, determining the number of positions at which the

identical nucleic acid base (for example, A, T, C, G, U, or I) occurs in both sequences to yield the

35   number of matched positions, dividing the number of matched positions by the total number of

positions in the window of comparison (that is, the window size), and multiplying the result by 100 to

yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a

characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that

has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more

usually at least 99 percent sequence identity as compared to a reference sequence over a comparison

window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides,

5   wherein the percentage of sequence identity is calculated by comparing the reference sequence to the

polynucleotide sequence which may include deletions or additions which total 20 percent or less of

the reference sequence over the window of comparison. The reference sequence may be a subset of a

larger sequence.

In addition to these polynucleotide sequence relationships, proteins and protein regions

10   encoded by recombinant HMPV are also typically selected to have conservative relationships, that is,

to have substantial sequence identity or sequence similarity, with selected reference polypeptides. As

applied to polypeptides, the term "sequence identity" means peptides share identical amino acids at

corresponding positions. The term "sequence similarity" means peptides have identical or similar

amino acids (that is, conservative substitutions) at corresponding positions. The term "substantial

15   sequence identity" means that two peptide sequences, when optimally aligned, such as by the

programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity,

such as at least 90 percent sequence identity, at least 95 percent sequence identity or more (for

example, 97, 98, or 99 percent sequence identity). The term "substantial similarity" means that two

peptide sequences share corresponding percentages of sequence similarity. Preferably, residue

20   positions that are not identical differ by conservative amino acid substitutions. Conservative amino

acid substitutions refer to the interchangeability of residues having similar side chains. For example,

a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and

isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a

group of amino acids having amide-containing side chains is asparagine and glutamine; a group of

25   amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino

acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having

sulfur-containing side chains is cysteine and methionine. Exemplary conservative amino acids

substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-

valine, and asparagine-glutamine. Abbreviations for the twenty naturally occurring amino acids used

30   herein follow conventional usage (Immunology - A Synthesis, 2nd ed., E.S. Golub & D.R. Gren, eds.,

Sinauer Associates, Sunderland, MA, 1991). Stereoisomers (for example, D-amino acids) of the

twenty conventional amino acids, unnatural amino acids such as α, α-disubstituted amino acids, N-

alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components

for polypeptides of the current disclosure. Examples of unconventional amino acids include: 4-

35   hydroxyproline, γ-carboxyglutamate, ε-N,N,N-trimethyllysine, ε-N-acetyllysine, O-phosphoserine,

N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω-N-methylarginine, and

other similar amino acids and imino acids (for example, 4-hydroxyproline). Moreover, amino acids

may be modified by glycosylation, phosphorylation and the like.

To select candidate viruses, the criteria of viability, attenuation and immunogenicity are

determined according to well-known methods. Viruses that will be most desired in immunogenic

5   compositions must maintain viability, have a stable attenuation phenotype, exhibit replication in an

immunized host (albeit at lower levels), and effectively elicit production of an immune response in a

recipient sufficient to elicit a desired immune response. The recombinant HMPVs are not only viable

and appropriately attenuated, they are more stable genetically *in vivo*--retaining the ability to

stimulate an immune response and in some instances to expand the immune response elicited by

10   multiple modifications, for example, induce an immune response against different viral strains or

subgroups, or to stimulate a response mediated by a different immunologic basis, for example,

secretory versus serum immunoglobulins, cellular immunity, and the like.

Recombinant HMPVs can be tested in various well-known and generally accepted *in vitro*

and *in vivo* models to confirm adequate attenuation, resistance to phenotypic reversion, and

15   immunogenicity. In *in vitro* assays, the modified virus (for example, a multiply attenuated,

biologically derived or recombinant HMPV) is tested, for example, for temperature sensitivity of

virus replication, that is, ts phenotype, and for the small plaque or other desired phenotype. Modified

viruses are further tested in animal models of HMPV infection. A variety of animal models have

been described and are summarized in various references incorporated herein. HMPV model

20   systems, including rodents and non-human primates, for evaluating attenuation and immunogenic

activity of HMPV candidates are widely accepted in the art, and the data obtained there from

correlate well with HMPV infection, attenuation and immunogenicity in humans.

In accordance with the foregoing description, the present disclosure also provides isolated,

infectious recombinant HMPV for use in immunogenic compositions. The attenuated virus which is

25   a component of an immunogenic composition is in an isolated and typically purified form. By

"isolated" is meant to refer to HMPV which is in other than a native environment of a wild type virus,

such as the nasopharynx of an infected individual. More generally, isolated is meant to include the

attenuated virus as a component of a cell culture or other artificial medium where it can be

propagated and characterized in a controlled setting. For example, attenuated HMPV can be

30   produced by an infected cell culture, separated from the cell culture and added to a stabilizer.

For use in immunogenic compositions, recombinant HMPV produced according to the

current disclosure can be used directly in formulations, or lyophilized, as desired, using lyophilization

protocols well known to the artisan. Lyophilized virus will typically be maintained at about 4°C.

When ready for use the lyophilized virus is reconstituted in a stabilizing solution, for example, saline

35   or comprising SPG, Mg^{++} and HEPES, with or without adjuvant, as further described herein.

HMPV-based immunogenic compositions contain as an active ingredient an

immunogenically effective amount of a recombinant HMPV produced as described herein. The

modified virus can be introduced into a host with a physiologically acceptable carrier and/or
adjuvant. Useful carriers are well known in the art, and include, for example, water, buffered water,
0.4% saline, 0.3% glycine, hyaluronic acid and the like. The resulting aqueous solutions may be
packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile

5   solution prior to administration, as mentioned above. The compositions may contain
pharmaceutically acceptable auxiliary substances as required to approximate physiological
conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and
the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium
chloride, sorbitan monolaurate, triethanolamine oleate, and the like. Acceptable adjuvants include

10   incomplete Freund's adjuvant, MPL™ (3-O-deacylated monophosphoryl lipid A; Corixa, Hamilton
IN) and IL-12 (Genetics Institute, Cambridge MA), among many other suitable adjuvants well known
in the art.

         Upon immunization with a recombinant HMPV composition, via aerosol, droplet, oral,
topical or other route, the immune system of the host responds to the immunogenic composition by

15   producing antibodies specific for HMPV proteins, for example, F and G glycoproteins. As a result of
the immunization with an immunogenically effective amount of a recombinant HMPV produced as
described herein, the host becomes at least partially or completely immune to infection by the
targeted HMPV or non- HMPV pathogen, or resistant to developing moderate or severe infection
there from, particularly of the lower respiratory tract.

20   The host to which the immunogenic compositions are administered can be any mammal
which is susceptible to infection by HMPV or a selected non- HMPV pathogen and which host is
capable of generating an immune response to the antigens of the immunizing strain. Accordingly,
methods are provided for creating immunogenic compositions for a variety of human and veterinary
uses.

25   The compositions containing the recombinant HMPV are administered to a host susceptible
to or otherwise at risk for HMPV infection to enhance the host's own immune response capabilities.
Such an amount is defined to be a "immunogenically effective dose." In this use, the precise amount
of recombinant HMPV to be administered within an effective dose will depend on the host's state of
health and weight, the mode of administration, the nature of the formulation, etc., but will generally

30   range from about 10^3 to about 10^7 plaque forming units (PFU) or more of virus per host, more
commonly from about 10^4 to 10^6 PFU virus per host. In any event, the formulations should provide a
quantity of modified HMPV of the current disclosure sufficient to elicit a detectable immune
response in the host patient against the subject pathogens.

         The recombinant HMPV can be combined with viruses of other HMPV serotypes or strains

35   to elicit a desired immune response against multiple HMPV serotypes or strains. Alternatively, an
immune response against multiple HMPV serotypes or strains can be achieved by combining
protective epitopes of multiple serotypes or strains engineered into one virus, as described herein.

Typically when different viruses are administered they will be in admixture and administered simultaneously, but they can also be administered separately. Immunization with one strain may immunize against different strains of the same or different serotype.

In some instances it may be desirable to combine the recombinant HMPV immunogenic

5   compositions with immunogenic compositions that induce immune responses to other agents, particularly other childhood viruses. In another aspect of the current disclosure the recombinant HMPV can be employed as a vector for protective antigens of other pathogens, such as RSV, by incorporating the sequences encoding those protective antigens into the recombinant HMPV genome or antigenome that is used to produce infectious virus, as described herein.

10   In all subjects, the precise amount of recombinant HMPV administered, and the timing and repetition of administration, will be determined using conventional methods based on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc. Dosages will generally range from about 10^3 to about 10^7 plaque forming units (PFU) or more of virus per patient, more commonly from about 10^4 to 10^6 PFU virus per patient. In any event, the formulations

15   should provide a quantity of attenuated recombinant HMPV sufficient to effectively stimulate or induce an anti- HMPV or other anti-pathogenic immune response, for example, as can be determined by hemagglutination inhibition, complement fixation, plaque neutralization, and/or enzyme-linked immunosorbent assay, among other methods. In this regard, individuals are also monitored for signs and symptoms of upper respiratory illness. As with administration to chimpanzees, the attenuated

20   virus of grows in the nasopharynx of recipients at levels approximately 10-fold or more lower than wild type virus, or approximately 10-fold or more lower when compared to levels of incompletely attenuated virus.

In neonates and infants, multiple administrations can be required to elicit sufficient levels of immunity. Administration should begin within the first month of life, and at intervals throughout

25   childhood, such as at two months, six months, one year and two years, as necessary to maintain an immune response against native (wild type) HMPV infection. Similarly, adults who are particularly susceptible to repeated or serious HMPV infection, such as, for example, health care workers, day care workers, family members of young children, the elderly, individuals with compromised cardiopulmonary function, can require multiple immunizations to establish and/or maintain immune

30   responses. Levels of induced immunity can be monitored by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to maintain desired levels of immune response. Further, different candidate viruses may be indicated for administration to different recipient groups. For example, an engineered HMPV expressing a cytokine or an additional protein rich in T cell epitopes may be particularly advantageous for adults

35   rather than for infants.

HMPV-based immunogenic compositions can be combined with viruses expressing antigens of another subgroup or strain of HMPV to elicit an immune response against multiple HMPV

subgroups or strains. Alternatively, the candidate virus can incorporate protective epitopes of multiple HMPV strains or subgroups engineered into one HMPV clone, as described herein.

The recombinant HMPV immunogenic compositions elicit production of an immune response that reduces or alleviates serious lower respiratory tract disease, such as pneumonia and

5   bronchiolitis when the individual is subsequently infected with wild type HMPV. While the naturally circulating virus is still capable of causing infection, particularly in the upper respiratory tract, there is a very greatly reduced possibility of rhinitis as a result of the vaccination. Boosting of resistance by subsequent infection by wild type virus can occur. Following immunization, there are detectable levels of host engendered serum and secretory antibodies which are capable of neutralizing

10   homologous (of the same subgroup) wild type virus *in vitro* and *in vivo*.

In one embodiment, recombinant HMPV candidates exhibit a very substantial diminution of virulence when compared to wild type virus that naturally infects humans. The virus is sufficiently attenuated so that symptoms of infection will not occur in most immunized individuals. In some instances the attenuated virus can still be capable of dissemination to nonimmunized individuals.

15   However, its virulence is sufficiently abrogated such that severe lower respiratory tract infections in the immunized host do not occur.

The level of attenuation of recombinant HMPV candidates can be determined by, for example, quantifying the amount of virus present in the respiratory tract of an immunized host and comparing the amount to that produced by wild type HMPV or other attenuated HMPV which have

20   been evaluated as candidate strains. For example, the attenuated virus will have a greater degree of restriction of replication in the upper respiratory tract of a highly susceptible host, such as a chimpanzee, compared to the levels of replication of wild type virus, for example, 10- to 1000-fold less. In order to further reduce the development of rhinorrhea, which is associated with the replication of virus in the upper respiratory tract, a useful candidate virus should exhibit a restricted

25   level of replication in both the upper and lower respiratory tract. However, the attenuated viruses must be sufficiently infectious and immunogenic in humans to elicit a desired immune response in immunized individuals. Methods for determining levels of HMPV in the nasopharynx of an infected host are well known in the literature and facilitated by the methods and compositions disclosed herein.

30   Levels of induced immunity provided by the immunogenic compositions of the current disclosure can also be monitored by measuring amounts of neutralizing secretory and serum antibodies. Based on these measurements, dosages can be adjusted or immunizations repeated as necessary to maintain desired levels of immune response. Further, different candidate viruses can be advantageous for different recipient groups. For example, an engineered recombinant HMPV strain

35   expressing an additional protein rich in T cell epitopes may be particularly advantageous for adults rather than for infants.

In another embodiment, the recombinant HMPV is employed as a vector for transient gene therapy of the respiratory tract. According to this embodiment the recombinant HMPV genome or antigenome incorporates a sequence that is capable of encoding a gene product of interest. The gene product of interest is under control of the same or a different promoter from that which controls

5   HMPV expression. The infectious recombinant HMPV produced by coexpressing the recombinant HMPV genome or antigenome with the N, P, L and other desired HMPV proteins, and containing a sequence encoding the gene product of interest, is administered to a patient. Administration is typically by aerosol, nebulizer, or other topical application to the respiratory tract of the patient being treated. Recombinant HMPV is administered in an amount sufficient to result in the expression of

10   therapeutic or prophylactic levels of the desired gene product. Representative gene products that may be administered within this method are preferably suitable for transient expression, including, for example, interleukin-2, interleukin-4, gamma-interferon, GM-CSF, G-CSF, erythropoietin, and other cytokines, glucocerebrosidase, phenylalanine hydroxylase, cystic fibrosis transmembrane conductance regulator (CFTR), hypoxanthine-guanine phosphoribosyl-transferase, cytotoxins, tumor

15   suppressor genes, antisense RNAs, and other candidate antigens.

The following examples are provided by way of illustration, not limitation. These examples describe the development of a novel reverse genetics system for the recovery of HMPV from cDNA, and the use of this system for construction of novel recombinant HMPV candidates. Thus, the subject matter of the current disclosure is illustrated by the following non-limiting Examples.

20
                                                    EXAMPLES

The mononegaviruses generally have a genome that comprises a single negative-sense strand of RNA tightly encapsidated in a nucleocapsid. There is a virally encoded polymerase that associates with the nucleocapsid and directs transcription of the genome by a process that initiates at

25   the 3′ end and copies the linear array of genes by a sequential stop-start mechanism that yields subgenomic mRNAs. Replication involves the synthesis of a complete positive sense copy called the antigenome, which in turn serves as the template for producing progeny genomes. Newly synthesized viral glycoproteins are transported to and accumulate at the plasma membrane. Progeny virus is formed when the nucleocapsid associates with these areas of modified plasma membrane and

30   acquires a lipid envelope by budding.

RSV is the leading cause of hospitalization for viral lower respiratory tract disease in infants and young children, followed by HPIV3 (Collins et al., 4th ed. In "Fields Virology," D. M. Knipe, P. M. Howley, Eds., Vol. 1, pp. 1443-1485, Lippincott-Williams and Wilkins Publishers, Philadelphia, 2001; Crowe et al., Vaccine 13:415-421, 1995; Marx et al., J. Infect. Dis. 176:1423-1427, 1997).

35   HPIV1 and HPIV2 are the principal etiologic agents of laryngotracheobronchitis (croup), and can also cause pneumonia and bronchiolitis (Chanock et al., 4th ed. In "Fields Virology," D. M. Knipe, P.
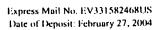
M. Howley, Eds., Vol. 1, pp. 1341-1379, Lippincott-Williams and Wilkins Publishers, Philadelphia, 2001). RSV and the PIVs also are important causes of respiratory tract disease in adults. The available evidence indicates that HMPV is also a significant agent of human respiratory tract disease, particularly in young infants and children but occurring in all age groups (Boivin *et al.* J. Infect. Dis.

5   186:1330-6, 2002).

HMPV replicates slowly and inefficiently in cell culture, rendering it relatively challenging to maintain and study in laboratory. In addition, trypsin reportedly must be present in the medium in order to activate infectivity of the virus, which complicates the lengthy incubations that are necessary to successfully propagate the virus. Indeed, the difficulty in propagating HMPV provides an

10   explanation for why it previously escaped detection as an important respiratory pathogen despite several decades of widespread cultivation of respiratory tract specimens in cell culture in many hospitals and research facilities across the world.

Methods for the production of RSV and PIV are known in the art, and compositions to induce an immune response against these viruses have been described (see Durbin *et al.*, Virology

15   235:323-332, 1997; Skiadopoulos *et al.*, J. Virol. 72:1762-1768, 1998; Skiadopoulos *et al.*, J. Virol. 73:1374-1381, 1999; Tao *et al.*, Vaccine 19:3620-3631, 2001; Durbin *et al.*, J. Virol. 74:6821-6831, 2000; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Patent Application Serial No. 09/458,813, filed December 10, 1999; U.S. Patent Application Serial No. 09/459,062, filed December 10, 1999; U.S. Provisional Application No. 60/047,575, filed May 23, 1997

20   (corresponding to International Publication No. WO 98/53078), U.S. Provisional Application No. 60/059,385, filed September 19, 1997; U.S. Provisional Application No. 60/170,195 filed December 10, 1999; and U.S. Patent Application Serial No. 09/733,692, filed December 8, 2000 (corresponding to International Publication No. WO 01/42445A2), each incorporated herein by reference). However, HMPV provides a contrasting situation in which there are no available reference reagents such as

25   monoclonal antibodies or available reference viral strains, and no established methods for the detection and analysis of HMPV. Knowledge concerning the molecular biology of HMPV has remained rudimentary and was previously based largely on extrapolation from other viruses. Because HMPV has only recently been identified and its characterization had been very incomplete, there were many challenges to generating suitably attenuated, immunogenic and genetically stable for

30   use in immunogenic and diagnostic compositions. To facilitate these goals, it is necessary to produce recombinant infectious HMPV from cDNA to serve as a starting point for manipulations that include the development and staged introduction of predetermined attenuating mutations; the deletion, modification or rearrangement of existing genes, the introduction of foreign genes, the swapping of protective antigens between strains, and the swapping of attenuating or other desired mutations

35   between HMPV strains and between HMPV and other related viruses, and to provide additional tools to generate immunogenic compositions, vectors and immunization methods. The present disclosure

describes the production of recombinant infectious HMPV from cDNA, and attenuating mutations with this cDNA, as well as the specific deletions and substitutions within existing genes, the introduction of heterologous nucleic acid sequences, and additional methods and compositions that are of use in methods designed to generate an immune response against HMPV.

5

Example 1

Determination of a Complete Consensus Sequence for the Genome of HMPV

The present Example provides the complete genomic sequence of a HMPV . The subject

10   viral strain for this analysis is strain CAN97-83 (Peret *et al.*, J. Infect. Dis. 185:1660-3, 2002), hereafter referred to as strain 83 or CAN83. The sequence was determined by direct analysis of uncloned RT-PCR products of viral RNA and thus represents a consensus or majority sequence of a viable virus. The virulence, and hence wild type status, of this virus was confirmed by the ability to induce respiratory tract disease signs in chimpanzees following intranasal inoculation, as described in

15   a following Example. The determination of a complete authentic wild type HMPV sequence is a necessary step in developing a system to produce HMPV of defined sequence and characteristics for use in immunogenic compositions and methods.

Cell Lines and Viruses

20   HEp-2 (ATCC CCL 23), Vero (ATCC CCL-81) and LLC-MK2 (ATCC CCL 7.1) cells were maintained in OptiMEM I (Invitrogen GIBCO) supplemented with 5% fetal bovine serum and, in some instances, gentamicin sulfate (50 µg/mL). BSR T7/5 cells are baby hamster kidney 21 (BHK-21) cells that have been transformed and constitutively express T7 RNA polymerase (Buchholz *et al.*, J. Virol. 73:251-9). They were maintained in Glasgow MEM supplemented with glutamine and

25   amino acids (Invitrogen) and 5% fetal bovine serum. The BSR T7/5 cells were subjected to geneticine (1 mg/ml) selection every second passage. Biological and recombinant HMPV were propagated in Vero, LLC-MK2, or BSR T7/5 cells in the absence of serum and the presence of 5 µg/ml of trypsin.

30   *Virion RNA Isolation*

Confluent monolayers of LLC-MK2 cells were infected with HMPV and incubated at 32°C in the presence of 5 µg/ml trypsin. 10-14 days post-infection, clarified supernatants were harvested. Virion RNA (vRNA) was isolated directly from the clarified supernatants using the QIAamp viral RNA purification kit (Qiagen) according to the manufacturer's instructions. Alternatively, virus was

35   purified by sucrose gradient centrifugation. Supernatant and infected cells were harvested by scraping with a rubber policeman, mixed with HEPES buffer and $MgSO_4$ at a final concentration of 50 mM

and 0.1 M, respectively, and then submitted to three cycles of freezing and thawing. The mixture was clarified by low-speed centrifugation and the virus was pelleted by centrifugation at 7000 rpm overnight at 4°C. The virus pellet was resuspended in OptiMEM (Invitrogen) containing 50 mM of HEPES buffer and 0.1 M of $MgSO_4$. The viral suspension was layered on a 30 to 60% sucrose

5 gradient and was centrifuged for 90 min at 26,000 rpm in an SW28 rotor. The upper virus-containing fraction was collected, diluted in TEN (10 mM Tris-HCl [pH 7.4], 0.1 M NaCl, 1 mM EDTA) and virus particles were pelleted by centrifugation at 25,000 rpm in an SW28 rotor for 90 min. The virus pellet was resuspended in TEN and vRNA was isolated directly from an aliquot using the RNeasy kit (Qiagen) according to the manufacturer's instructions.

10
Reverse Transcription, Polymerase Chain Reaction and Nucleotide Sequencing

vRNA was reverse transcribed using specific primers generated from the published partial sequence of HMPV strain 00-1. In most cases these failed to yield RT-PCR products, presumably reflecting nucleotide sequence differences between these two strains. Therefore additional primers

15 were designed based on the regions between ORFs in the 00-1 sequence that, by analogy to other mononegaviruses, would likely contain cis-acting signals that would be more highly conserved between HMPV strains. This strategy provided RT-PCR products for strain 83 that, upon sequence analysis, provided information for designing strain 83-specific primers. Purified RNA was mixed with 50 pmol of different primers in a final volume of 14 μl in water and incubated for 5 min at 70°C

20 and 25 min at 60°C for denaturing and annealing steps. Then, 5 μl of 5X first-strand buffer II, 1 μl of 10 mM deoxynucleoside triphosphates (dNTPs), 2 μl of 0.1 M dithiothreitol, 1 μl of RNaseOUT (40 U/μl; Invitrogen) and 2 μl of Superscript II reverse transcriptase (Invitrogen) were added to the reaction. Synthesis of cDNA by RT was conducted at 44°C for 1 h and 30 min at 51°C to minimize the formation of RNA secondary structures. PCR was carried out on the reverse transcribed cDNA

25 product using 50 pmol each of specific forward and reverse primers, 5 μl of 10X Pfx amplification buffer, 1 μl of dNTP mixture, 3 μl of $MgSO_4$ and 1 μl of Platinum Pfx DNA polymerase in a total volume of 50 μl. The PCRs were conducted on a Peltier Thermal Cycler (PTC-200; MJ Research) as follows: 3 min at 94°C and then 40 s at 94°C, 40 s at 55°C and 1 min/Kb at 68°C for 30 cycles and a final extension reaction for 7 min at 68°C. RT and PCR primers designed from the 00-1 strain

30 sequence to prime within ORFs usually were not successful generating RT-PCR products from the 83 strain, presumably reflecting sequence differences relative to the 00-1 strain. Therefore, primers were designed from the semi-conserved sequence motifs flanking the viral ORFs (see Figure 8), which resulted in the successful generation of cDNA. The nucleotide sequences of cDNA products were determined by direct sequence analysis of the RT–PCR products using a ABI 3100 sequencer with

35 the Big-Dye terminator ready reaction kit v1.1 (Applied Biosystems). Finally, the sequence was assembled from overlapping RT–PCR products.
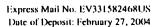
To map and sequence the 3' end of the HMPV genome, vRNA was converted into cDNA and amplified using the 3' RACE System for Rapid Amplification of cDNA Ends (Invitrogen, Inc.) as specified by the manufacturer. Briefly, vRNA was polyadenylated at its 3'-end using poly A polymerase (Invitrogen, Inc.) followed by first-strand cDNA synthesis primed with oligo (dT) and

5   PCR using an HMPV specific reverse primer and a forward UAP primer supplied with the kit. Then, RACE products were used as template for a nested-PCR using an internal HMPV specific reverse primer and the AUAP primer supplied with the kit to improve the specificity of the reaction. Finally, the amplified cDNA nested-PCR products were sequenced directly.

To map and sequence the 5' end of the HMPV genome, vRNA was processed by first-strand

10   cDNA synthesis, terminal transferase tailing, and PCR amplification as specified by the 5' RACE System for Rapid Amplification of cDNA 5' end Version 2.0 (Invitrogen, Inc.) using the AAP primer supplied with the kit. Then, the amplified cDNA RACE products were used as template for a nested-PCR using an internal HMPV specific primer and the AUAP primer supplied with the kit to improve the specificity of the reaction. Finally, the amplified cDNA nested-PCR products were sequenced

15   directly.

The 5' and 3' ends of the HMPV genome also were confirmed by genomic RNA ligation and RT-PCR followed by sequencing. Briefly, vRNA, isolated from sucrose gradient purified virus using the RNeasy kit (Qiagen), was ligated in presence of 10 U of T4 RNA ligase (Epicentre) for 3 h at 25°C and 3 h at 37°C. A cDNA corresponding to the junction of the ligated ends was amplified by

20   RT-PCR using HMPV specific primers. The RT-PCR products were used as template for a nested-PCR using HMPV internal specific primers to improve the specificity of the reaction. Finally, the nested-PCR products were cloned into a blunt vector (pSTBlue-1; Novagen) and 20 clones were sequenced, thereby confirming the length, content and sequence of the two previously-uncharacterized ends of the HMPV genome.

25   The complete sequence of HMPV strain 83 was determined to be 13335 nucleotides in length, and is set forth in Figures 37A-37D. This sequence was determined directly from uncloned RT-PCR products and thus represents the authentic majority, consensus sequence of a virulent, wild type virus. This discovery provides a necessary step in the development of a reverse genetics system for recovering complete infectious virus and recombinantly engineered derivatives from cloned

30   cDNAs. A partial sequence had been published representing a portion of the genome of HMPV strain 00-1 (van den Hoogen et al., Virology 295:119-132, 2002).

A genomic map for HMPV 83 is shown in FIG. 1. Compared to RSV, the prototype pneumovirus, HMPV lacks the NS1 and NS2 genes and contains a significant difference in gene order. Specifically, the SH-G gene pair precedes the F-M2 gene pair in RSV, whereas SH-G follows

35   F-M2 in HMPV. Identification of HMPV genes was made based on the presence of ORFs whose predicted protein products exhibit significant amino acid sequence relatedness with counterpart ORFs in RSV (N, P, M, F, M2-1 and L) or, in cases where sequence relatedness was unclear, gene
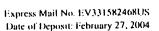
identifications were made by genome position and by the characteristics of the predicted protein (M2-
2, G, SH). In these analyses, RSV was chosen for comparison because all of its proteins have been
directly identified and in most cases functions have been assigned. In a number of cases, the size of
the predicted HMPV protein was substantially different from that of its RSV counterpart. For

5   example, the putative HMPV SH protein was 180% larger than that of RSV (179 amino acids
compared to 64) and the HMPV G protein was 27% shorter (219 amino acids compared to 298) and
lacked certain characteristic structural features such as the conserved cysteine noose (Johnson *et al.*,
Proc. Natl Acad.Sci. USA 84:5625-9, 1987) and CX3C chemokine domain (Tripp *et al.*, Nat. Med.
2:732-8, 2001). Even the putative HMPV P and L proteins exhibited significant differences in length,

10   being 22% larger and 7% shorter, respectively, than the proposed RSV counterparts.

        The ends of the HMPV genome were mapped and sequenced. The deduced sequences of the
3′ leader and 5′ trailer regions are shown in FIG. 2. This analysis ruled out the possibility that one or
more additional genes might follow the L gene or precede the N gene, comparable to the NS1 and NS
genes of RSV. Comparison of the leader and trailer regions of HMPV 83 with that of APV

15   (Randhawa *et al.*, J. Virol. 71:9849-9854, 1997) and RSV (Mink *et al.*, Virology 185:615-624, 1991)
showed a high degree of sequence identity between the first 22-23 nucleotides at each end, consistent
with the idea that these conserved sequences represent important functional domains. In the case of
RSV, it was directly demonstrated that the first 11 nucleotides of the RSV genome represent the core
promoter essential for both transcription and RNA replication (Fearns *et al.*, J. Virol. 76:1663-1672,

20   2002).

        Thus, the published partial sequence for strain 00-1 appears to lack the core element of both
the genomic and antigenomic promoters, and probably lacks any HMPV promoter whatsoever. It is
noteworthy that, within the first 12 nucleotides at either end of the HMPV genome, there were at least
one (3′ leader) or two (5′ trailer) sequence differences compared to the other pneumoviruses. Thus,

25   the expedient of using the termini of APV or RSV to supplement the incomplete HMPV 00-1
sequence by constructing a chimeric molecule would not have yielded an authentic HMPV sequence
at either end. While the functional significance of these particular differences have yet to be fully
elucidated, studies with RSV showed that any point mutations within the genomic promoter affected
transcription or RNA replication or both, and in most cases these effects were severe (Fearns *et al.*, J.

30   Virol. 76:1663-1672, 2002). In any event, for the development of a genetic system to engineer
recombinant HMPV for use in immunogenic compositions, it is critical to begin with a verified
authentic wild type sequence, as provided herein. This sequence was used as a starting point;
confirmation that the sequence indeed is authentic and encodes a functional virus is provided by the
recovery of infectious, wild type-like recombinant virus. This demonstration is provided in examples

35   below.

        Another important feature of mononegavirus genomic RNA is that the 3′ and 5′ ends
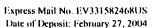typically exhibit terminal complementarity, meaning that the two ends are complementary when
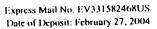
aligned in an antiparallel fashion. This reflects the presence of a conserved sequence at each end that probably reflects a conserved promoter core. This terminal complementarity is shown for HMPV 83 and APV in FIG. 3. For typical mononegaviruses, the two ends exhibit a high degree of complementarity for at least the first 12 nucleotides and often more, and typically within this region

5   the complementarity is exact or has at most one mismatch. As shown in FIG. 3, the APV genome indeed has an exact match for the first 13 nucleotides whereas, surprisingly, the HMPV 83 genome has two adjacent mismatches. This further illustrates that extrapolation from other pneumoviruses to develop a HMPV genetic system based on an incomplete sequence could not have yielded an authentic HMPV sequence.

10   The sequence of HMPV 83 contained a number of differences compared to that published for strain 00-1. As examples, FIG. 4 shows that the SH ORF terminated four codons earlier in the 83 sequence than in that for strain 00-1, yielding a shorter SH protein. Also, the G ORF in the HMPV 83 sequence contained a deletion that shortened the G protein by 17 amino acids, and the intergenic region between the F and M2 genes was 13 nucleotides for 83 instead of 41 nucleotides. The

15   significance of differences such as shown in FIG. 4 can be directly evaluated with the reverse genetics system of this current disclosure.

The percent amino acid sequence identity between the predicted proteins of HMPV strain 83 and those of other pneumoviruses is shown in FIG. 5. It should be noted that a subsequent Example provides a more extensive comparison between strain 83 and another HMPV strain, 75, that forms

20   part of the current disclosure, demonstrating the extent of sequence diversity between and within the proposed HMPV subgroups. It is noteworthy that, among all of the pneumoviruses shown in FIG. 5, reverse genetics systems for the recovery of complete infectious virus have been reported only in the case of bovine RSV (BRSV) and human RSV subgroup A. This is despite that fact that PVM has been known for almost 65 years and has been the subject of extensive molecular analysis beginning

25   in the late 1980's, and despite the fact that APV has been known for almost 25 years and also is the subject of molecular analysis. The fact that reverse genetics systems are not yet available despite considerable interest in their development illustrates the difficulty of producing such systems. As has already been noted, HRSV and BRSV differ from HMPV with regard to the lack of two genes (NS1 and NS2) and the rearrangement of other genes (SH and G). HRSV and BRSV also differ extensively

30   from HMPV with regard to amino acid sequence identity of the predicted HMPV proteins. Three of the potential HMPV proteins (SH, G, and M2-2), share only 6-15% sequence identity with their putative HRSV counterparts, whereas low sequence identity values (for example, less than 25% identity) are generally considered at best to correspond to remote homologs or, alternatively, to proteins that are unrelated. Conversely, the highest value for percent identity is only 46%, in the

35   case of the L protein. Thus, HMPV is quite distinct from any virus for which a reverse genetics system for the recovery of virus has been developed. It should be further noted in this context that the function of a protein in HMPV cannot be reliably predicted by extrapolation from results obtained for

a related protein of a related virus, not even for a closely related virus. Briefly, there is initial
uncertainty in deducing proteins from nucleotide sequence. For example, despite the reported
existence of an M2-2 ORF in APV, the available evidence suggests that this ORF is not expressed
(Ahmadian et al., J. Gen. Virol. 80:2011-2016, 1999), whereas it clearly is expressed in RSV and has
5   been shown to have a role in regulating RNA synthesis (Bermingham and Collins, Proc. Natl. Acad.
Sci. USA 96:11259-11264, 1999; Jin et al., J. Virol. 74:74-82, 2000). Thus, the presence of an ORF
is not proof alone of significance. As another example, in the Filovirus family, both Ebola and
Marburg viruses express a protein called VP30 that is highly related between the two viruses.
However, these two proteins have different functions (Weik et al. J. Virol. 76:8532-9, 2002;
10   Muhlberger et al., J. Virol. 73:2333-42, 1999). These are but two examples of the uncertainty in
predicting the existence and function of a protein based on nucleotide sequence alone. This highlights
the importance of direct, relevant assays as provided hereon for the demonstration of protein function.
These are presented in the current disclosure in two forms: (i) the development of a mini-replicon that
is capable of transcription and replication when supplied with appropriate viral proteins, and
15   importantly (ii) the development of a system for the recovery of complete infectious virus completely
from cDNA, which provides the basis for analysis of the function of all elements of the genome and
its encoded RNA and protein products and infectious virus.

The amino acid sequence of the predicted M protein of HMPV 83 was used to estimate the
phylogenetic relationships between HMPV and other members of the Paramyxovirus family, as
20   illustrated in FIG. 6. As shown in this figure, the Paramyxovirus family consists of two subfamilies
Pneumovirinae and Paramyxovirinae, herein referred to as pneumoviruses and paramyxoviruses,
respectively. These subfamilies are further divided into genera, the genus of HMPV being the
Metapneumoviruses. A system for producing infectious recombinant virus did not exist previously
for any member of this genus. This analysis provided further evidence of evolutionary distance and
25   distinction from pneumoviruses such as RSV and, in particular, from paramyxovirus such as Sendai,
measles, mumps, human parainfluenza, and Newcastle disease viruses that constitute the more well-
known and more extensively-characterized members of the Paramyxovirus family.

Example 2

30   **Assembly of a Full-Length HMPV Antigenomic cDNA Clone Containing a GFP Transcription
Cassette and Recovery of Infectious Recombinant HMPV**

The complete, authentic, consensus sequence (FIGS. 37A-37D) determined for HMPV
strain 83 provided the basis for designing a system for producing infectious recombinant virus. FIG. 7
illustrates the construction of a cDNA clone encoding the complete antigenome of HMPV strain 83
35   designed from the complete consensus sequence. Three separate cloned subgenomic fragments were
created: fragment 1 contains the putative N, P and M genes and is bordered on the upstream (left)
side by an added promoter for bacteriophage T7 RNA polymerase (T7pr) and on the right hand side
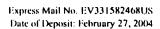
by an *Nhe*l site that was added to the putative N-F intergenic region as a marker. The T7 promoter was designed to add three nonviral G residues to the 5' end of the antigenome, a configuration chosen to improve the efficiency of the T7 promoter. Fragment 2 included the putative F, M2, SH and G genes and is bordered on the upstream side by the added *Nhe*l site and on the downstream side by a

5 naturally-occurring *Acc*65I site. The *Nhe*l site serves as a marker to distinguish between cDNA-derived and biologically-derived HMPV. Fragment 3 consists of the putative L gene followed by half of the hepatitis delta virus ribozyme (HDVribo) bordered by an *Rsr*II site that occurs naturally within the ribozyme. The sequence of each cloned cDNA fragment was confirmed. The vector for cloning and expressing the HMPV 83 antigenome cDNA is pBSKSII, which contains the hepatitis delta virus

10 ribozyme followed by a terminator for T7 RNA polymerase (T7t) and was derived from vector p3/7 that was used previously for the recovery of recombinant HPIV1, HPIV2 and HPIV3 (Durbin *et al.*, Virology 235:323-332, 1997; Schmidt *et al.*, J. Virol. 74:8922-9, 2000; Skiadopoulos *et al.*, J. Virol. 77:270-9, 2003; Newman *et al.*, Virus Genes 24:77-92, 2002). This vector was modified by the insertion of a polylinker containing *Ava*I, *Aat*II, *Nhe*l, and *Acc*65I sites, which in turn served to accept

15 the fragment 1, 2 and 3 restriction sites. Specifically, the polylinker consists of the following sequence, *CCCGGG*GACGTCCTAGCTAGCTAGGGTACCCCGCTCGAG*CGGTCCG* (SEQ ID NO: 33; *Sma*I and *Rsr*II sites italicized), and was inserted between the *Sma*I and *Rsr*II sites of the vector p3/7 of Durbin *et al.* (Durbin *et al.*, Virology 235:323-332, 1997). The final cDNA encodes the complete 13335-nucleotide HMPV 83 antigenome containing three added nonviral G residues at

20 the 5' end. The complete pHMPV-83 recombinant plasmid ("antigenome plasmid") contains 16333 bp.

It was anticipated that the poor growth and lack of established reference reagents and assays for HMPV could complicate the recovery and identification of recombinant HMPV recovered from cDNA. Therefore, the strategy was adopted to modify the antigenomic cDNA to insert a foreign

25 marker gene encoding a detectable label, whereby expression of the marker gene could be readily monitored. The gene that was chosen encodes the well-known marker or label GFP, a cnidarian protein whose presence can be visualized by green fluorescent emission when observed under a fluorescent microscope. The GFP cDNA was the Enhanced GFP cDNA of Clonetech, Inc. Expression of a detectable marker in this manner, directly linked with HMPV production, makes it

30 possible to monitor expression of GFP by recombinant HMPV in living cells without loss of viability or sterility, and to thereby detect and quantify the recovery of rHMPV at all stages of transfection and passage.

Among the obstacles to this strategy were the lack of direct information on HMPV transcription and the lack of direct mapping of gene boundaries. It therefore was necessary to identify

35 sequence that, when attached to the foreign ORF, would render it capable of being expressed during the HMPV transcriptional program. It also was necessary to identify a site within the HMPV genome that could accommodate the insertion of a foreign gene without disrupting HMPV growth. The
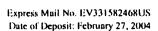
transcription of a typical mononegavirus genome initiates at or near the 3′ end and proceeds by a
sequential start-stop mechanism by which the individual genes are transcribed into individual,
separate mRNAs. Typically, mononegavirus transcription is guided by short sequence motifs that
flank the viral genes. For example, the genes of RSV, which is the most thoroughly characterized

5   pneumovirus, contain a highly-conserved 10-nucleotide GS signal on the upstream end of each gene
and a semi-conserved 12- to 13-nucleotide GE signal at the downstream end of each gene. These
motifs were first noticed when RSV mRNAs were isolated from infected cells and their termini were
directly mapped and sequenced. It is thought that the GS signal directs initiation of transcription of
the individual gene, and the GE signal directs polyadenylation and termination. The polymerase

10   remains bound to the template and crosses the intergenic region to resume transcription at the next
gene. In the case of RSV, the analysis of the mRNA ends showed that the arrangement of genes was
unexpectedly complex. Specifically, the GS signal of the L gene was found to differ from that of the
others, and furthermore was found to be located within its upstream neighbor rather than downstream
of it. As another example, the M2-1 and M2-2 ORFs were found to be contained in a single mRNA

15   rather than two. Also, there was no consistency of spacing of the RSV GS and GE signals with
respect to the ORFs, such that there were instances where the GS signals overlapped the ORFs or,
alternatively, were separated from the ORFs by as many as 85 nucleotides. Similarly, certain RSV
GE signals overlapped the respective ORF or were separated by as many as 173 nucleotides.
Furthermore, the spacing between the RSV genes was irregular, ranging from the overlapped genes

20   mentioned above to ones separated by as many as 56 nucleotides. This indicates the uncertainty of
predicting gene boundaries based on the locations of putative ORFs and potential sequence motifs.
However, the methods of this current disclosure provide for the direct identification of appropriate
transcription signals and insertion sites.

The complete sequence of HMPV strain 83 was examined for potential GS and GE motifs,

25   as shown in FIG. 8. These motifs were identified on the basis of being located between ORFs and by
exhibiting partial sequence conservation among themselves. For example, the putative HMPV GS
motif has the sequence GgGAcAAgTgaaaATG, where the nucleotide assignments in upper case are
conserved in each of the putative genes. In sharp contrast to RSV, the putative GS signals were found
in close proximity to the start of their respective putative ORFs, with the putative initiation ATG

30   codon located at positions 14-16 relative to each putative GS signal. It should be noted that the L
ORF was preceded by two potential GS sequences, either of which was a reasonable fit for the
proposed consensus sequence. One of these is shown in FIG. 8, while the second is located 18 nt
upstream (7102-GGGCAAAACAGCATCC). Typically mononegavirus genes each have a single GS
site, and it is not known which one of these is functional for the HMPV L gene. However, this can

35   now be readily determined by the methods of this current disclosure, by mapping the 5′ end of the
putative L mRNA by RT-PCR, or by testing each potential GS signal for functionality in a mini-

replicon system, or by destroying each potential signal in the complete antigenome and evaluating each mutant for virus recovery and efficiency of growth.

The putative GE motif has the sequence AGTtaattaAAAA, where the upper case assignments are conserved in each of the putative genes. The limited number of highly-conserved

5   nucleotide assignments in these motifs made their identification speculative, particularly since not a single HMPV signal had been directly identified to use as a benchmark. While these conserved motifs appear to contain the core of the GS and GE signals, it would not be surprising to find that either sequence as described here might be shortened at one or both ends by one or several nucleotides without complete loss of activity, or that one or more additional nucleotides added to either side

10   might affect the activity of either signal. As an indication of the uncertainty of deducing transcription signals from sequence data alone, van den Hoogen *et al.* noted two different types of consensus sequences between genes, one of which corresponds to that noted above (van den Hoogen *et al.*, Virology 295:119-132, 2002). In this regard, these authors concluded that they could clearly identify this particular consensus sequence for two genes, F and L (although, as noted above, there would

15   appear to be two candidates to be the GS signal for the L gene), found "variants" in several other genes, failed to detect a counterpart for the G gene, and noted that "another [different] repeated sequence...was found downstream of each of the hMPV ORFs except G", raising further uncertainty as to which, if any, of these sequence motifs might function as a transcription signal. Thus, it was important to directly identify transcription signals that could render a foreign sequence competent for

20   expression by HMPV.

In order to directly identify sequence signals that can confer gene expression, the 16-nucleotide putative GS sequence and 13-nucleotide putative GE sequence of the N gene were attached to a cDNA containing the GFP ORF. In this configuration, the ATG present in the GS signal replaced that of the GFP ORF. As shown in FIG. 9, this transcription cassette was inserted into the

25   HMPV antigenomic cDNA following nucleotide 41, resulting in an antigenome in which the GFP gene was placed first in the 5′ to 3′ order. In the corresponding HMPV-GFP genome, the GFP gene would be the most promoter-proximal of the genes. The complete sequence of the antigenomic rHMPV-GFP cDNA (FIGS. 38A-38D) and flanking plasmid regions was confirmed by sequence analysis.

30   Initially four of the ORFs of HMPV, namely the putative N, P, L and M2-1 ORFs, were selected to be expressed in separate plasmids to complement recovery. This initial recovery protocol focused on proteins predicted to be "internal" proteins (as opposed to components of the envelope). Examination of the HMPV genome identified ORFs whose potential products share 41%, 31%, 36%, and 46% identity with the RSV N, P, M2-1 and L proteins, respectively (FIG. 5), and these ORFs

35   were selected as candidates for inclusion. The putative M2-1 HMPV ORF also was included in the recovery because it is a possible internal protein, and studies with RSV, Ebola virus and Marburg virus indicate that possible M2-1 counterparts can be involved in RNA synthesis. In this regard it is

noted that the presumed M2-1 counterparts appear to play different roles in Ebola and Marburg viruses despite their high degree of relatedness (Weik *et al.* J. Virol. 76:8532-9, 2002; Muhlberger *et al.*, J. Virol. 73:2333-42, 1999). Also, the putative HMPV M2-1 protein contains a Cys-7/Cys-15/Cys-21/His-25 motif (numbered according to the complete deduced HMPV M2-1 amino acid

5   sequence, designated herein the Cys3-His1 motif) that is a potential zinc binding domain that might indicate a role in interacting with nucleotides or nucleic acid. On the other hand, the putative M2-2 ORF was not included in the initial recovery system, and there is evidence from APV that a seemingly-corresponding M2-2 ORF is not expressed (Ahmadian *et al.*, J. Gen. Virol. 80:2011-2016, 1999).

10   The ORFs for the G, F and SH proteins of HMPV were not included in the initial recovery system, because these ORFs were predicted to encode transmembrane surface proteins--based on the presence of putative hydrophobic signals and anchors in the deduced amino acid sequences. Notably, the predicted HMPV F protein shares 36% amino acid sequence relatedness to that of RSV. The percent amino acid identity for the putative G and SH proteins between HMPV and RSV are 15% and

15   6%, respectively. Despite this substantial departure in primary structure, other features of the amino acid sequences, such as the presence of potential glycosylation sites support the identification of these proteins herein. It is generally acknowledged in the field that the envelope proteins do not have a known role in genome transcription and RNA replication. This does not preclude the possibility that the expression of these additional ORFs might facilitate recovery, perhaps by increasing the

20   efficiency of virion assembly. However, an important aspect of the current disclosure involves identification of a minimal complement of support plasmids necessary for HMPV recovery. Once minimal recovery elements were shown to be successful, additional embodiments can now be constructed and evaluated to optimize the efficiency of recovery. For example, although the expression of the M2-1 ORF from a separate plasmid is shown herein to be non-essential for

25   recovery, it may nonetheless be a desired complement that could function to influence transcription or RNA replication or some other aspect of HMPV replication. Accordingly, expression plasmids were constructed in which the N, P, L and M2-1 ORFs were placed under the control of a T7 transcription promoter and terminator.

        Support plasmids encoding putative HMPV nucleocapsid and polymerase proteins were

30   prepared as follows (FIG. 10): to prepare a support plasmid expressing the putative N protein, vRNA was subjected to RT-PCR using a positive sense primer designed to hybridize at beginning of the putative N ORF (nucleotides 55 to 76 in the HMPV genome) and a reverse primer designed to hybridize at the end of the putative N ORF (nucleotides 1239 to 1220 in the HMPV genome). The PCR product was digested with *Xho*I and subjected to a partial digestion with *Afl*III, which was done

35   because the putative N ORF contains a naturally-occurring *Afl*III and a partial digest would yield some product that had been cleaved at the terminal site and not at the internal site. The products were into pTM1 (Durbin *et al.*, Virology 235:323-332, 1997; Durbin *et al.*, Virology 234:74-83, 1997;

Elroy-Stein *et al.*, Proc. Natl. Acad. Sci. USA. 86:6126-30, 1989) that was digested with *Nco*I (which leaves overhangs compatible with *Afl*III) and *Xho*I, and clones containing the complete putative N ORF were selected and the sequence confirmed.

To construct a support plasmid containing the putative P ORF of HMPV (pT7-P) (FIG. 10),
5   vRNA was subjected to RT-PCR using a positive sense primer designed to hybridize at beginning of the putative P ORF (nucleotides 1263 to 1279 in the HMPV genome) and a reverse primer designed to hybridize at the end of the putative P ORF (nucleotides 2147 to 2132 in the HMPV genome). The PCR product was digested with *Afl*III and *Xho*I and cloned into pTM1 that was digested with *Nco*I and *Xho*I. In the final cloned recombinant plasmid, the sequence of the HMPV insert and flanking
10 plasmid regions was confirmed by sequence analysis..

A comparable putative M2-1 support plasmid for HMPV (pT7-M2-1) was generated (FIG. 10). vRNA was subjected to RT-PCR using a positive sense primer designed to hybridize at beginning of the putative M2-1 ORF (nucleotides 4724 to 4740 in the HMPV genome) and a reverse primer designed to hybridize at the end of the putative M2-1 ORF (nucleotides 5287 to 5272 in the
15 HMPV genome). The PCR product was digested with *Afl*III and *Bam*HI and cloned into pTM1 that was digested with *Nco*I and *Bam*HI. In the final cloned recombinant plasmid, the sequence of the HMPV insert and flanking plasmid regions was confirmed by sequence analysis.

A support plasmid containing the putative L ORF of HMPV (pT7-L; FIG. 10) was made by RT-PCR amplification of two overlapping fragments and cloned separately into pTM1 vector. The
20 first fragment was amplified by RT-PCR using a positive sense primer designed to hybridize at the beginning of the putative L ORF (nucleotides 7133 to 7157 in the HMPV genome) and a reverse primer designed to hybridize downstream to a unique *Avr*II (nucleotides 10892 to 10897 in the HMPV genome) restriction site (nucleotides 11037 to 11018 in the HMPV genome), and the second using a positive sense primer designed to hybridize upstream to the unique *Avr*II site (nucleotides
25 10845 to 10866 in the HMPV genome) and a reverse primer designed to hybridize at the end of the putative L ORF (nucleotides 13150 to 13124 in the HMPV genome). Then, the first fragment was treated by *Bam*HI and the Klenow fragment of the *E. coli* polymerase I to generate a blunt end, digested by *Avr*II and cloned into pTM1 that was treated by *Nco*I and Klenow enzyme and digested by *Avr*II. Finally, the second fragment of the putative L ORF was digested by *Avr*II and *Xho*I and
30 cloned in the intermediate construct digested by the same enzymes, leading to the final construct pT7-L. In the final cloned recombinant plasmid, the sequence of the HMPV insert and flanking plasmid regions was confirmed by sequence analysis.

To provide an initial functional evaluation of these support plasmids, a mini-replicon system was developed. A cloned cDNA was constructed so that its expression from a T7 promoter yielded a
35 negative-sense mini-replicon RNA, or minigenome, that contained, in 3′-to 5′ order: the leader end of the HMPV genome and adjoining putative N GS signal followed by a negative sense copy of the ORF encoding bacterial chloramphenicol acetyl transferase (CAT), followed by the putative L GE

signal and the trailer end of the HMPV genome. The correct 3' end was generated by a HDV
ribozyme as described above. In this minigenome, the CAT gene would be under the control of
putative HMPV GS and GE signals, and would be expressed as a subgenomic mRNA. The
minigenome plasmid was transfected into HEp-2 cells together with the N, P, M2-1 and L support

5   plasmids with simultaneous coinfection by vTF-7, a vaccinia virus recombinant that expresses T7
RNA polymerase. 48 h post transfection, intracellular RNA was isolated and analyzed by Northern
blot hybridization using a double stranded probe specific to the CAT cDNA. This analysis
demonstrated that coexpression of the N, P and L genes with the minigenome plasmid yielded RNA
species of the appropriate size to represent progeny minigenomes, antigenomes, and subgenomic

10 CAT mRNA. In this assay, the further addition of the M2-1 gene did not alter the profile of RNA
products. This indicated that in this assay N, P and L coexpression was sufficient to reconstitute RNA
replication and transcription. These findings are in sharp contrast to RSV, where the expression of
M2-1 is necessary to achieve efficient synthesis of mRNA (Collins *et al.*, Proc. Natl. Acad. Sci. USA
93:81-85, 1996; Fearns and Collins, J. Virol. 73:5852-64, 1999). These preliminary results do not

15 rule out any role for M2-1 in HMPV RNA synthesis, since it is possible that M2-1 might have one or
more functions that are not required for minigenome replication and transcription but might be
essential to launch infection by complete recombinant virus. The current disclosure provides the
methods to expeditiously evaluate the role of M2-1 (and M2-2) in viral recovery, gene expression,
and replication.

20   To test the ability to generate infectious recombinant HMPV from cDNA, the full-length
HMPV-GFP antigenome plasmid was cotransfected with the N, P, M2-1 and L plasmids into BSR
T7/5 cells, which are baby hamster kidney-21 (BHK-21) cells that constitutively express T7 RNA
polymerase (Buchholz *et al.* J. Virol. 73:251-59, 1999). Transfections were done in 6-well dishes
with 5 µg of antigenomic plasmid, 2 µg each of N and P support plasmid, and 1 µg each of M2-1 and

25 L support plasmid per well. Transfections were done with SuperFect (Qiagen) or Lipofectamine
2000 (Invitrogen) in medium without trypsin or serum. One day post transfection trypsin was added
to 5 µg/ml. Also, to maintain the cells in an active state, they typically were split and reseeded at a
1:3 ratio one or two days post transfection. In another, alternative permutation, fresh trypsin was
added one day post transfection, the cells were incubated for one or more hours, the cells were

30 scraped into the medium, and the total suspension was passaged to fresh LLC-MK2 or Vero cells.
Approximately 12-24 h later the medium was replaced with fresh medium containing 5 µg/ml trypsin
(unless otherwise noted, 5 µg/ml trypsin was the concentration that was routinely used).

          When examined by fluorescent microscopy on successive days post-transfection, green cells
were visualized that initially consisted of scattered isolated cells and subsequently formed small foci

35 that consisted of two or more cells and exhibited cytopathic effect consistent with HMPV. When the
transfection monolayer was split into new cultures, single green cells were visualized within one day
and developed over successive days into multicellular syncytia. The expression of GFP was

monitored by fluorescent microscopy and photographed 8 days post-transfection. The nature of the
cytopathology was indistinguishable from that produced by biologically-derived HMPV. The
recovered virus replicated in LLC-MK2 cells with kinetics and cytopathogenicity consistent with
HMPV.

5   The ability to monitor the infection in live cells without compromising sterility can be used
to expeditiously examine conditions to improve growth. For example, it was found that the
replenishment of trypsin by the addition of further amounts during incubation resulted in a greater
number of infected cells. In a preliminary experiment, the addition of fresh trypsin to a concentration
of 2.5 or 5 µg/ml at two-day intervals resulted in the highest level of GFP expression and virus
10   spread. However, the higher level also was associated with increased syncytium formation, which has
the potential to cause more rapid cell death and decreased yield. Further experimentation, combined
with assay of released infectious virus (an assay that also is facilitated by the expression of the GFP
marker), will serve to identify the condition of incubation and level of trypsin that is optimal for the
production of infectious virus. The most optimal schedule found to date has been to add 5 µg/ml
15   fresh trypsin at intervals of 2 or 3 days.

           The expression of GFP by cells infected with the recovered virus provided evidence that the
virus was cDNA-derived and was not contaminating biologically-derived HMPV. Further
identification of the virus as recombinant HMPV was made in two ways, by immunofluorescence and
by detection of the Nhel restriction site marker. In the immunofluorescence assay, antibodies from
20   hamsters that had been infected with biologically-derived HMPV reacted specifically with cells
infected with the putative recovered rHMPV-GFP. For this assay, BSR T7/5 cells were transfected
for the recovery of recombinant HMPV-GFP as described above. Four days later the cells were split
again at a ratio of 1:3 and incubated on a coverslip for nine more days. Incubations were in the
presence of 5 µg/ml trypsin. The cells were fixed with 80% acetone for 15 min at 4°C, followed by
25   incubation with serum from hamsters that had been infected with HMPV, followed by incubation
with goat antibodies that were specific to hamster IgG and had been labeled with the fluorescent tag
Alexa-488. The same field of cells was visualized by confocal microscopy with visible light and
under conditions for fluorescence. This demonstrated strong fluorescence that was specific to foci in
HMPV-infected cultures.

30   In a related assay series, RT-PCR was performed to amplify nucleotides 2719 to 3894
(numbered according to their position in the wild type HMPV sequence exclusive of GFP) in the
rHMPV-GFP genomic RNA (FIG. 12A). This RT-PCR procedure yielded a product of the expected
size when the template was RNA from cells infected with biologically-derived HMPV or
recombinant HMPV-GFP, but only in the latter case was the cDNA cleaved by Nhel, confirming the
35   presence of the added Nhel site specific to the recombinant antigenomic cDNA from which the
recombinant virus was derived. Amplification of RT-PCR products was dependent on the addition of
RT, indicating that the template was indeed viral RNA and not contaminating DNA. Infectious

recombinant HMPV-GFP also was recovered when the M2-1 support plasmid was omitted from the

panel of support plasmids. Although these results suggest that M2-1 is not required for recovery, it is

possible that M2-1 is expressed from the antigenome plasmid, as was found in the case of RSV

(Collins et al. Virology 295: 251-255, 1999). Also, it may be that co-expression of M2-1 will have

5   some qualitative or quantitative effect on recovery. This is suggested by the observation that, in a

typical experiment, three times more infectious virus was recovered in the presence of M2-1. It is

believed that the minimal complement of proteins required for recovery of HMPV is N, P and L.

       The expression of the inserted GFP coding sequence also was characterized by Northern blot

hybridization. Cells were infected with biologically-derived HMPV83, rHMPV, rHMPV-GFP, or

10   were mock infected, and total intracellular RNA was isolated three days later and analyzed by

Northern blot hybridization with double-stranded DNA probes specific to the GFP or M genes. As

shown in FIG. 12B, the GFP-specific probe hybridized only with RNA from rHMPV-GFP-infected

cells (lane 4), and detected an abundant RNA band of the appropriate size to be the predicted 746-nt

(exclusive of polyA) GFP mRNA transcribed from the inserted transcription cassette. This showed

15   that the putative HMPV GS and GE transcription signals indeed functioned in the context of the

foreign ORF to direct the efficient synthesis of a monocistronic GFP RNA. The GFP probe also

hybridized to several larger RNAs that were of low abundance and appeared to represent GFP-N,

GFP-N-P and GFP-N-P-M read-through mRNAs, as well as to a large, faint band that was of the

appropriate size to contain rHMPV-GFP genome and antigenome RNA. The very low levels of read-

20   though mRNAs indicated that termination at the end of the GFP transcription cassette was very

efficient, and hence an HMPV GE signal indeed had been correctly identified. Analysis with the M-

specific probe identified in each of the viruses bands of the appropriate sizes to be the predicted 853-

nt monocistronic M mRNA as well as bands representing the P-M and N-P-M read-through mRNAs.

The M probe also detected the genome and antigenome RNA band for each virus, which in the case

25   of rHMPV-GFP would be 748 nt larger than HMPV83 or rHMPV due to the presence of the GFP

transcription cassette (FIG. 12B, lanes 6, 7 and 8).

       To test the ability to recover recombinant HMPV that lacked the GFP gene, plasmid

expressing the HMPV antigenome depicted in FIG. 11 but lacking the GFP insert was transfected

into BRS T7/5 cells together with N, P, M2-1 and L support plasmids. The recovery of recombinant

30   HMPV was confirmed by the detection of viral antigen in single cells and in foci by indirect

immunofluorescence assay as described above.

       The recovered recombinant HMPV strain 83 was compared to the biologically-derived strain

83 isolate with regard to the efficiency of multi-cycle replication in vitro. LLC-MK2 cells were

inoculated with 0.01 plaque forming units (PFU) per cell and incubated at 32°C in the presence of 5

35   μg/ml trypsin. Aliquots of the medium were taken at 24 h intervals and flash frozen. The aliquots

were subsequently diluted in medium containing fresh trypsin and analyzed in parallel by plaque

assay to determine the infectivity titer. As shown in FIG. 13, the recombinant HMPV replicated

efficiently over the 10 day period tested. The efficiency of replication of rHMPV was essentially indistinguishable from that of its biologically –derived counterpart. The ability to recover a wild type-like virus entirely from cloned cDNA showed that the sequences used to construct these cDNAs are fully functional, and thus define an authentic, functional, prototypic wild type HMPV and its encoded

5   proteins. This is noteworthy, since these viruses exhibit a high frequency of mutation (and thus a sequence cannot be assumed to be functional). The rHMPV antigenomic cDNA had been sequenced in its entirety following construction, as noted above, with the result that its sequence was confirmed to be exactly as designed. Thus, this experiment established (i) the exact genomic sequence of a prototypic HMPV, and (ii) conditions for recovering cloned cDNAs representing this sequence. This

10   verified prototypic system provides the basis for the expedited recovery of other strains of HMPV, as well as for the systematic modification of HMPV via changes introduced into the cloned cDNA intermediate. Finally, the ability to precisely reconstruct a wild type recombinant HMPV indicates the robustness and precision of the methods disclosed herein. This is further demonstrated by the ability to recovery numerous engineered derivatives described in subsequent examples.

15   Next, recombinant HMPV-GFP was compared to rHMPV with regard to the efficiency of multi-cycle growth *in vitro* to evaluate the effect of the addition of a transcription cassette. As shown in FIG. 14, rHMPV-GFP replicated over the tested 10-day period with an efficiency that, at times, was reduced more than 10-fold compared to rHMPV. However, at the end of the 10 day period the final titers were very similar. Thus, a foreign insert can be accommodated by HMPV without a

20   drastic effect on *in vitro* replication, such that a recombinant virus containing such an insert can be feasibly manufactured. It also is possible that such an insert will prove to be attenuated *in vivo*, as can now be expeditiously determined in experiments employing experimental animals that were identified as supporting HMPV replication in experiments described in a subsequent Example.

25   **Example 3**
      The Development of Recombinant HMPV Derivatives Containing Pre-determined Mutations
                          Specifying Desired Phenotypic Changes
              The ability to produce infectious rHMPV from cDNA provides the basis for the planned introduction of mutations into infectious HMPV to develop recombinant viral candidates possessing

30   desirable characteristics for use in immunogenic compositions, such as temperature-sensitivity, cold adaptation, host range restriction, improved replication *in vitro*, reduced reactogenicity, increased safety, increased antigen expression, increased genetic or phenotypic stability, broader immunogenic coverage, increased immunogenicity, and attenuation. Mutations might be devised that have advantages in other applications as well, such as to yield increased antigen expression and/or to

35   facilitate preparation of purified viral protein. Each antigenome can be assayed for the ability to direct the recovery of infectious virus, and recovered mutant viruses can be evaluated for *in vitro* affects on growth and cytopathogenicity, temperature sensitivity, plaque morphology, and other characteristics.

Appropriate mutant viruses can then be evaluated for growth efficiency, antigen expression, temperature sensitivity, plaque morphology, cytopathogenesis, attenuation, pathogenicity, tropism, genetic and phenotypic stability, immunogenicity, safety, and protective efficacy in cell culture and predictive animal models for HMPV activity in human subjects, including mice, hamsters, cotton

5   rats, and non-human primates, as well as in clinical studies.

One type of desirable mutation involves the deletion of sequence from the HMPV genome. For example, each of the HMPV genes or ORFs or parts thereof can be systematically deleted either in its entirety or in part, either alone or in combination with other genes or ORFs, to obtain virus with improved properties, particularly for use within immunogenic compositions. Exemplary genes or

10   ORFs include the SH, G, M2-2 and M2-1 ORFs, but can involve any gene or ORF or genome region or part thereof, including non-ORF sequences such as noncoding genes or extragenic regions or cis-acting signals in whole or in part . As an example of the strategy of deleting entire genes, antigenomic cDNAs were designed in which the putative SH and G ORFs, each with their surrounding-set of putative GS and GE signals, were deleted singly or together (FIG. 15). To make

15   these constructs, the wild type GFP was modified to insert *BsiW*1 and *BsrG*1 sites at positions 5459 and 6099 (numbered relative to the complete antigenome exclusive of GFP, a convention that will be followed throughout this document). These positions are within the M2-SH and SH-G intergenic regions, respectively. Cleavage at either of these two sites creates the overhang GTAC, which also is the case for the naturally-occurring *Acc*651 site located at position 6959, within the G-L intergenic

20   region (FIG. 15). Thus, the presence of these three compatible restriction sites flanking the SH and G genes facilitate deletion of either or both genes by cleavage with the appropriate pairs of enzymes followed by religation. This resulted in cDNAs encoding rHMPV-GFP antigenomes that lacked the SH (ΔSH) or G (ΔG) genes or both (ΔSH/G). Each antigenomic cDNA was evaluated for the ability to direct the recovery of infectious virus in BSR-T7 cells in the presence of the N, P, L and M2-1

25   support plasmids. In each case, infectious virus was readily and successfully recovered. These could then be evaluated with regard to kinetics and efficiency of growth *in vitro* and in experimental animals to define advantageous properties including attenuation. The presence of the expected deletion in each virus was confirmed by RT-PCR analysis of the SH-G region of each recovered genome, as shown in FIG. 16. Also, limited nucleotide sequencing of the RT-PCR products

30   confirmed the sequences at the junctions of the deletions.

The rHMPV-GFP ΔSH, ΔG and ΔSH/G mutants were compared with rHMPV-GFP and rHMPV with regard to the efficiency of multi-cycle replication LLC-MK2 cells following infection with an input of 0.01 PFU per cell (FIG. 17A). In this experiment, the gene-deletion mutants contained the GFP marker gene and thus should be compared to rHMPV-GFP. Remarkably, rHMPV-

35   GFP containing the deletion of a single gene, either SH or G, replicated 3- to 10-fold more efficiently than rHMPV-GFP alone. Thus, these genes are not essential for replication *in vitro* and, indeed, their loss improved replicative fitness *in vitro*. This may reflect a growth advantage due to the shorter

length of the genome and loss of a transcriptional unit, although it also is possible that the absence of the SH or G protein might somehow improve growth. This can readily be investigated by the methods disclosed herein by ablating translation of SH and/or G by making nucleotide substitutions that remove translational start codons and introduce termination codons, thereby ablating expression of

5   the protein while maintaining the gene number, the genome length, and the number of transcribed mRNAs. rHMPV-GFP lacking both SH and G replicated with an efficiency similar to that of complete rHMPV-GFP, suggesting as one interpretation that whatever gain might be made from deleting genes and shortening the genome was counteracted by a slight loss of replicative fitness due to the absence of both SH and G. Thus, production of an HMPV immunogenic composition might be

10   improved by deletion of SH or G, but deletion of both might not be optimal. Also, deletion of either gene might increase the ability of rHMPV to accommodate one or more added foreign genes. Given the efficient replication of these viruses *in vitro*, it will now be possible to evaluate the effects of these gene deletions on HMPV replication, immunogenicity, and pathogenesis in experimental animals and human volunteers.

15   A second, parallel set of ΔSH, ΔG, and ΔSH/G mutants was made in an rHMPV strain 83 backbone lacking the GFP marker gene. Each of the viruses from this second set of mutants was readily recovered and propagated *in vitro*. These could be evaluated with regard to kinetics and efficiency of growth *in vitro* and in experimental animals to define advantageous properties including immunogenicity and attenuation. While the presence of the GFP gene can facilitate characterization

20   of mutant viruses *in vitro* and *in vivo*, the GFP gene preferably would not be included in candidates for developing immunogenic compositions. Hence, it is advantageous to be able to expeditiously produce mutants with or without GFP, or both, as appropriate.

     The set of rHMPV ΔSH and ΔG viruses lacking the GFP marker gene were evaluated for replication in vivo (FIG. 17B). Golden Syrian hamsters were infected intranasally with biologically-

25   derived HMPV83, rHMPV, rHMPVΔSH, rHMPVΔG, or rHMPVΔSH/G. Six animals from each group were sacrificed three or five days later and the nasal turbinates and lungs were harvested, homogenized, and analyzed by limiting dilution to determine the viral titers (FIG. 17B). There were several instances where differences in titer were observed between days 3 and 5, the most notable being the higher titers of HMPV 83, rHMPV and rHMPVΔSH in the lungs on day 3 versus day 5.

30   However, in general the titers for the two days followed a consistent pattern. Recombinantly-derived HMPV replicated in vivo with an efficiency similar to that of its biologically-derived parent HMPV83. Thus, rHMPV appeared to have wild type-like growth properties in vivo as well as in vitro and represents a suitable starting point for developing attenuated derivatives as candidate vaccines.

     Interestingly, the replication of HMPV was not significantly reduced by deletion of the SH

35   gene (FIG. 17B). Indeed, the ΔSH virus replicated marginally better than its wild type counterpart, perhaps due to the shorter genome length and reduced gene number. In contrast, deletion of the G gene resulted in a 2.9 (day 5) to 3.2 (day 3) log_{10} decrease in replication in the nasal turbinates, and a

0.3 (day 5) to 2.3 (day 3) log₁₀ decrease in the lungs. The rHMPVΔSH/G double-deletion virus exhibited a similar marked reduction in virus titer. Thus, these mutant viruses are highly attenuated in vivo.

In order to evaluate possible immunogenicity and protective efficacy, the rHMPV,

5   rHMPVΔSH, rHMPVΔG and rHMPVΔSH/G viruses were administered intranasally to additional hamsters (FIG. 17C). Serum samples were taken 27 days later and analyzed for the ability to neutralize HMPV infectivity in vitro (FIG. 17C). This showed that the highly attenuated ΔG and ΔSH/G viruses induced high titers of HMPV-neutralizing serum antibodies even though they were highly attenuated. On day 28 post immunization, the animals were challenged intranasally with wild

10   type HMPV, and the animals were sacrificed three days later and nasal turbinates and lungs were harvested and virus titers were determined by limiting dilution (FIG. 17C). This showed that no challenge virus replication could be detected in animals that had been infected with rHMPV or rHMPVΔSH. Interestingly, animals that had been infected with rHMPVΔG or rHMPVΔSH/G also did not have detectable challenge virus replication in the lungs, and only a low level of challenge

15   virus replication was detected in the nasal turbinates. Thus, despite their strongly attenuated nature, the rHMPVΔG and rHMPVΔSH/G viruses were immunogenic and highly protective against HMPV challenge. These viruses represent promising vaccine candidates. In particular, it was remarkable that deletion of the G gene would yield a promising vaccine candidate, since the G protein is presumed to be an attachment protein important in initiating infection, and the corresponding deletion in HRSV

20   yielded a virus that did not replicate in mice (Teng et al, Virology 289, 283-296, 2001) and was over attenuated in humans (Karron et al., Proc. Natl. Acad. Sci. USA 94: 13961-13966).

The deletion or otherwise modification of intergenic regions of HMPV offers an important strategy for achieving improved phenotypic properties. For example, the intergenic regions of HMPV strain 83 range in length from 2 nt (N/P) to 190 nt (G/L). Previous work with RSV indicated

25   that plaque size was decreased if a single intergenic region was longer than 100 nt in length (Bukreyev et al., J. Virol. 74:11017-26, 2000). Two of the naturally-occurring intergenic regions of HMPV strain 83 are greater than 100 nt in length, namely SH/G (124 nt) and G/L (190 nt). Thus, reducing the length of these or other intergenic regions offers a strategy to improve replication *in vitro*, while increasing intergenic length offers a strategy for attenuating the virus, as desired.

30   As another example, recombinant HMPV was recovered in which the M2-2 ORF was silenced (FIG. 18A). The proposed M2-1 ORF initiates at nucleotide position 4724 and terminates at position 5287. The proposed M2-2 ORF potentially initiates at positions 5235 or 5247 and terminates at 5450, and thus overlaps the M2-1 ORF. To create a mutant HMPV in which the M2-2 ORF is silenced, single nucleotide substitutions were introduced at positions 5236 and 5248, which removed

35   the two potential translational start codons without changing the amino acid coding assignment in the overlapping M2-1 ORF (FIG. 18A). A third nucleotide substitution was made at position 5272, which introduced a translational stop codon into the M2-2 ORF. In addition, nucleotides 5289-5440 were

deleted, which deleted most of the M2-2 ORF. These changes were introduced into the wild type

rHMPV antigenomic cDNA as well as into the rHMPV-GFP antigenomic cDNA. In each case, the

ΔM2-2 virus was recovered successfully from cDNA. The rHMPVΔM2-2 and rHMPV-GFPΔM2-2

viruses were compared to their respective parents, rHMPV and rHMPV-GFP, with regard to multi-

5   step growth kinetics *in vitro* (FIG. 18B). This showed that each ΔM2-2 virus replicated somewhat

less efficiently than its rHMPV-GFP parent at many of the time points, although by the end of the

experiment at 13 hours, the titers were very similar. These mutations are of interest for vaccine

design, since they do not greatly affect growth in vitro (which is necessary for vaccine manufacture)

and are likely attenuating in vivo. They are evaluated in the hamster model noted in Fig. 17B and C,

10   and described more completely in a subsequent Example below.Northern blot hybridization was then

used to monitor the synthesis of intracellular RNA by the ΔM2-2 virus. As shown in FIG. 18C, the

synthesis of genome and antigenome by the ΔM2-2 virus was not greatly different than that of wild

type HMPV. However, the ΔM2-2 virus directed a level of mRNA synthesis that, when normalized to

the amount of genome from replicate gel lanes, was up to 8.7-fold that of wild type HMPV. The up-

15   regulation of protein synthesis without a concomitant increase in virus replication would be highly

desirable for a vaccine virus. This phenotype resembles, but is not identical to, that of the ΔM2-2

mutant of human RSV. For RSV, the ΔM2-2 mutant exhibited a delay in the accumulation of mRNA

that was not observed here, and exhibited a delay and reduction in the accumulation of genome and

antigenome that also was not observed for HMPV. Further unanticipated differences between the

20   HMPV and RSV are described herein, namely the ability to disrupt the HMPV M2-1 ORF, or to

delete M2-1 and M2-2 altogether, without loss of viral viability for HMPV whereas either mutation

was lethal for RSV.

In another application, illustrated in FIG. 19, a series of single amino acid substitutions was

introduced into the cysteine-histidine motif present in the predicted M2-1 protein of rHMPV-GFP.

25   This "Cys3-His1" motif consists of three cysteine residues at M2-1 amino acid positions 7, 15, and

21, and a histidine residue at position 25 (FIG. 19). This motif is exactly conserved in RSV and other

pneumoviruses. In RSV, the integrity of this motif is essential for the transcriptional processivity and

anti-termination function of M2-1, and is essential for the recovery of infectious recombinant virus

(Hardy and Wertz J. Virol. 74:5880-5885, 2000; Tang *et al.*, J. Virol. 75:11328-11335, 2001). The

30   amino acid substitutions introduced into the M2-1 protein of rHMPV-GFP were as follows: Cysteine-

7-Serine (C7S), Cysteine-15-Serine (C15S), and Histidine-25-Serine (H25S). As controls, two

additional mutants were made involving amino acids that are found in the vicinity of the motif but are

not considered to be part of the motif: Tyrosine-9-Serine (Y6S) and Asparagine-25-Serine (N25S).

Each of these mutants was readily recovered as infectious virus. The C7S, Y19S, C15S, N16S, H25S

35   and ΔM2-1 mutants of rHMPV-GFP were evaluated for multi-cycle growth in LLC-MK2 cells in

parallel with rHMPV and rHMPV-GFP, as shown in FIG. 20. The mutants exhibited a range of

growth efficiency relative to the rHMPV-GFP parent, with the Y9S mutant replicating more

efficiently, the C7S mutant replicating comparably, and the others replicating somewhat less efficiently. Nonetheless, with the exception of the H25S mutant, the final titers at 13 days were mostly comparable to that of the rHMPV-GFP parent.

In another mutation involving the M2-1 ORF of rHMPV-GFP, the ATG translational start site was changed to a TAG termination codon, as shown in FIG. 21, resulting in the mutant designated rHMPV-GFPΔM2-1. The next ATG codon is located far down the ORF, at codon 134 out of 187 codons, and hence this mutation should ablate synthesis of M2-1. In this same mutant, additional termination codons were introduced closely downstream in each of the three reading frames to further preclude possible ribosomal entry and passage down the ORF. The HMPV-GFP-ΔM2-1 mutant was readily recovered as infectious virus.

The ability to rapidly and effectively recover this panel of mutants in the M2-1 ORF was a further demonstration that the cDNA-based recovery system is a robust method for expeditiously developing rHMPV variants bearing predetermined changes. The finding that a number of these mutants replicated more efficiently that their direct parent rHMPV-GFP suggests that such mutants would be valuable for use in immunogenic compositions, since improved replication *in vitro* would greatly facilitate the production of rHMPV and also might be an improved source of HMPV antigen for a protein-based immunogenic composition. In these embodiments, rHMPV-GFP was used as a parent because the expression of the GFP marker facilitates *in vitro* characterization. These forms also can be directly evaluated in experimental animals, and the presence of the marker would facilitate analysis of possible effects on virus localization *in vivo*. In addition, non-GFP versions can readily be generated, as was done above for the ΔSH, ΔG and ΔSH/G viruses, which would be appropriate for clinical evaluation. The finding that the Cys3His1 motif, and the M2-1 ORF altogether, are not required for efficient growth *in vitro* was completely unanticipated, particularly since with the Cys3-His1 motif and M2-1 protein were previously shown to be essential for the recovery of infectious RSV (Tang *et al.*, J. Virol. 75:11328-11335, 2001). In addition, it was independently confirmed that mutations in the Cys3-His1 motif of RSV, or silencing the M2-1 ORF of RSV, prevented the recovery of recombinant RSV. Although the Cys3-His1 motif is not required for efficient HMPV replication in vito, it is reasonable to anticipate that this motif and this protein have been conserved in HMPV for functional reasons and do contribute to some aspect of HMPV replication or interaction with its host. Thus, it is reasonable to anticipate that some of these mutations will be found to be attenuating *in vivo* and useful for inclusion in a live attenuated HMPV immunogenic composition.

In another exemplary embodiment the HMPV M2 gene was deleted altogether from rHMPV-GFP, including its GS and GE signals and both the M2-1 and M2-2 ORFs (FIG. 22A). The resulting rHMPV-GFP-ΔM2(1+2) virus was readily recovered and propagated *in vitro*. The ΔM2-2, ΔM2-1, and ΔM2(1+2) viruses were compared with wild type HMPV with regard to the efficiency of multi-cycle replication in LLC-MK2 and Vero cells (FIG. 22B, upper and lower panels, respectively).

This experiment was performed with versions of the viruses that express GFP in order to facilitate visualization of growth, but the expression of GFP was not otherwise relevant to the experiment. In LLC-MK2 cells, each of the M2 mutant viruses replicated more than 10-fold less efficiently than wild type. In contrast, in Vero cells each of the M2 deletion viruses grew to a final titer that equaled or

5   exceeded that of wild type HMPV. One of the important differences between LLC-MK2 and Vero cells is that the latter lack the structural genes for type I interferon. Thus, this result suggested that that the M2 deletion viruses were more sensitive to interferon than wild type HMPV. In addition, the observation that the M2 deletion viruses replicated efficiently in Vero cells is important, since this is a cell substrate that is acceptable for preparing vaccines for humans.

10   To directly evaluate the interferon-sensitivity of the M2 deletion viruses, Vero cells were pre-treated overnight with a range of concentrations of type I interferon in order to induce an antiviral state. The ability of the ΔM2(1+2) and ΔM2-2 viruses to replicate was evaluated in the interferon-treated cells compared to wild type HMPV and wild type HRSV. Each of the viruses used in this experiment expressed GFP for the purpose of monitoring the infections, but the presence of GFP was

15   not otherwise relevant to the results. Cell monolayers were infected at an moi of 1 PFU per cell (or a multiplicity of infection (moi) of 0.01 in the case of RSV, reflecting its more efficient growth) and incubated for 4 days. Control monolayers were mock-interferon-treated, infected and processed in parallel. The media supernatants were harvested and analyzed by plaque assay to determine virus titers. The fold reduction between each interferon-treated culture and its corresponding mock-

20   interferon-treated control was calculated and is shown in FIG. 22C. Thus, the replication of wild type rHMPV-GFP were reduced from 5-fold to 1680-fold by increasing amounts of interferon, compared to mock-interferon-treated controls. In comparison, the ΔM2(1+2) and ΔM2-2 viruses were more sensitive and were reduced 19-fold and 13-fold, respectively at the lowest concentration and were completely inhibited at the highest concentration. Viruses that exhibit increased sensitivity to

25   interferon typically are attenuated in vivo. Thus, these results suggest that these viruses are candidates to be attenuated derivatives.

               Another exemplary embodiment of the current disclosure involves changing gene order to achieve attenuation, increase antigen expression, or achieve some other desirable phenotype. In a subsequent Example, the F protein of HMPV is identified as a major protective antigen, and the G

30   and SH are also identified as virion surface proteins, which makes them likely protective antigens. In the wild type gene order, SH, G and F are located at gene positions 6, 7 and 4, respectively. It is anticipated that HMPV transcription will exhibit a polar gradient, in which promoter-proximal genes are expressed more efficiently than downstream ones. Thus, placement of putative protective antigen genes such as SH, G, and F proximal to the promoter, either singly, as a pair, or as a triplet, will result

35   in more efficient expression. In this application, the genes can be moved to this position from their wild type location, such that the virus retains a single copy of each, or the genes can be inserted as a second copy, such that the virus now expresses two copies of one or more of the HMPV protective

antigens. The change in gene expression due to the relocation of genes or to the addition of a second copy can have other desirable effects, such as increased or decreased growth *in vitro* or *in vivo*.

Exemplifying this strategy of "shifting" gene position for developing HMPV derivatives with improved properties, the position of the SH-G gene pair was altered in rHMPV-GFP (FIG. 23A). In one construct, designated "Order #1", the SH-G gene pair was moved from its position following the M2 gene to a position preceding the F gene. This results in a gene order in that region of the genome that mimics that of RSV, namely: M-SH-G-F-M2-L. Hence, the positions of four genes were altered in "Order 1", namely SH and G (each moved upstream by two positions) and F and M2 (each moved downstream by two positions). Accordingly, any gene or combination of genes can be moved within the genome, obtaining changes in gene expression that can provide desirable phenotypes including increased antigen expression, improved growth *in vitro*, and attenuation. As shown in FIG. 23B, the kinetics and magnitude of replication of the mutant called RSV Order #1 was indistinguishable from that of its direct parent rHMPV-GFP. This showed that changes in gene order can readily be achieved, resulting in infectious virus that replicates efficiently *in vitro* and can be evaluated for desirable properties such as attenuation or improved antigen expression *in vivo*.

As another permutation shown in FIG. 23A, the SH-G pair was moved to be upstream of the F gene in rHMPV-GFP, and a second copy of the pair was inserted, resulting in the construct "Order # 2". Thus, this construct contains two copies of the SH and G genes, which are potential protective antigens. In this case, the F-M2 gene pair has been moved a total of four positions downstream relative to the viral promoter, and the L gene is now two positions further downstream compared to the parental wild type HMPV. Yet another strategy of "gene shifting" involves moving the presumed protective antigen G and F genes from their normal positions as the 7th and 4th genes, respectively, in the HMPV gene map to promoter-proximal positions. Each gene can be moved individually or as a pair, as shown in FIG. 24A. In the case of the constructs shown in FIG. 24A, the promoter-proximal insertion site will be the same as was used for insertion of the GFP transcription cassette to make the rHMPV-GFP construct (FIG. 9), from which efficient expression has been demonstrated. While these represent preferred signals and insertion sites, other signals and insertion sites can readily be evaluated and used by the methods of this current disclosure. Preferred insertion sites would be ones in proposed intergenic regions or regions of genes that are outside of the ORFs and putative transcription signals and thus more likely to accommodate engineering without affecting HMPV replication. A subsequent Example provides identification of genome regions that are poorly conserved between divergent strains of HMPV, suggesting that they are less likely to be essential, which provides further guidance to identify preferred insertion sites. The transcription cassette should preferably be designed so that, following its insertion into the antigenomic cDNA clone, the inserted ORF is flanked by a set of GS and GE signals and each gene is the backbone similarly is flanked by a set of transcription signals. In the gene-shift constructs shown in FIG. 24A, insertion of the G and/or F gene into a promoter proximal position was accompanied by deletion of the corresponding copy

from the normal antigenome position: in instances where G was shifted to the first or second position, nucleotides 6101-6960 were deleted to delete G from its natural location, and in instances where F was shifted, nucleotides 3053-4710 were deleted to delete the naturally-occurring copy of F. Analysis of the multi-step growth kinetics of these viruses *in vitro* (FIG. 24B) showed that each

5   replicated somewhat less efficiently than the wild type parent, although the final titers of the single-gene shift mutants (rHMPV-G1 and F1) were identical to that of the rHMPV parent, while the double-gene shift mutants were only slightly lower.

A further set of exemplary viruses were constructed in which the G and F genes were inserted into the promoter proximal positions, but the copies of the genes in their natural positions were left undisturbed, as shown in FIG. 25A. Thus, rHMPV+G1 and +F1 contains two copies of the

10 indicated gene (note that the symbol "+" in the designation indicates that the genes were added rather than "shifted"). Similarly, rHMPV+G1F2 and +F1G2 contained an additional copy each of G and F in the indicated position, and rHMPV+G1F2F3 contained an additional copy of G and two additional copies of F in the indicated positions. Interestingly, the multi-cycle growth kinetics of these viruses

15 were somewhat reduced compared to their rHMPV parent (FIG. 25B), but among themselves the various addition mutations grew with similar kinetics. Thus, while the addition of one gene was somewhat attenuating, as had also been observed with GFP, the further addition of one or two more genes had little effect, even in the case of the relatively large F gene. The level of replication in this particualr experiment had not plateaued, and the final virus yields were not determined. In these

20 particular constructs, the added genes were of the same virus strain. However, it also should be feasible to incorporate one or more genes from a different strain, such as one possessing antigenic differences. Thus, for example, a rHMPV+G1F2 virus could be made that expresses F and G pairs representing two different strains, resulting in a single bivalent recombinant virus that would elicit a broader immune response.

25 The presence and intactness of the additional genes in the genome of recovered rHMPV+G1F23 were investigated by RT-PCR performed on total RNA from recovered virus following three passages in Vero cells. RT-PCR was performed with a forward primer hybridizing to the HMPV leader region and a reverse primer hybridizing to N sequence, which yielded a single, major band of approximately 4.1 kb, the appropriate size to contain the tandem G1F2F3

30 supernumerary genes (not shown). The sequence of the supernumerary G1F2F3 genes could not be determined directly by RT-PCR consensus sequencing of viral RNA because primers specific to G or F would prime on each of the two copies of G and three copies of F, precluding analysis of each gene individually. Therefore, the supernumerary genes were first amplified by RT-PCR in three separate, overlapping segments. The first RT-PCR segment used a forward (positive-sense) primer (which also

35 served as the RT primer) from the leader region and a reverse primer from the F gene (840 nt downstream of the F GS signal): although this latter primer would prime on each copy of F, priming from the promoter-proximal copy (F2, numbered as in rHMPV+G1F23) would be the most efficient

in combination with this forward primer, and yielded a ~1.6 kb fragment that contained the leader,

the G1 gene, and about 840 bp of the F2 gene, and was purified by gel electrophoresis for sequence

analysis. The second segment was amplified by RT-PCR with an RT/forward primer located about

670 nt downstream of the F GS, and a reverse primer located 554 nt downstream of the F GS:

5   although the forward primer would prime in all three copies of F and the reverse primer in all three

copies of F, priming in F2 and F3 would be the only combination to result in successful amplification

of a PCR fragment (~1.5 kb) that contained the downstream 978 bp part of the F2 gene, and a 554 bp

upstream part of F3, and was purified by gel electrophoresis for sequence analysis. The third

fragment was generated with an RT/forward primer from the upstream end of F (14 nt downstream

10   the F GS signal) and a reverse primer from the upstream end of N: the forward primer would prime in

all three copies of F, but priming in F3 would be the most efficient and would yield a ~1.75 kb

product that contained most of the F3 gene, and 120 bp of the N gene, and was purified by gel

electrophoresis for sequence analysis. This provided an RT-PCR consensus sequence of the G1F2F3

supernumerary genes that was free of mutations, and all GS/GE signals and intergenic sequences

15   were correct, indicating that these added genes were stably recovered in recombinant virus.

To determine the effect of the gene additions on viral gene expression, cells were mock-

infected or infected with biologically-derived HMPV83, rHMPV, or rHMPV+G1F23, and total

intracellular RNA was isolated three days later and subjected to Northern blot analysis with double

stranded probes to the F, G or M genes (FIG. 25C). The F probe detected a major band of the

20   appropriate size to be the 1644-nt F mRNA, as well as a fainter band of genome and antigenome,

which in the case of rHMPV+G1F23 would be 4008 nt larger compared to HMPV83 or rHMPV due

to the presence of the three added gene copies (FIG. 25C, lane 4). There were only trace amounts of

readthrough mRNAs detected with the F probe. Phosphorimager analysis indicated that the amount of

genome plus antigenome for rHPMV+G1F23 compared to HMPV83 and rHMPV was 0.9 and 0.5,

25   respectively (corrected for the two extra copies of the F gene present in rHMPV+G1F23). In

comparison, the relative amount of F mRNA for rHMPV+G1F23 compared to HMPV83 and rHMPV

was 6 in each case. Normalized to the respective value of genome plus antigenome, this corresponded

to a 6.6- to 12-fold higher level of F mRNA for rHMPV+G1F23 compared to HMPV83 and rHMPV.

Northern blot analysis with the G probe detected, for each of the three viruses, a band of the

30   appropriate size to be the 711-nt G mRNA as well as bands corresponding to SH-G and M-SH-G

readthrough mRNAs and a band corresponding to antigenome and genome RNAs (FIG. 25C, lanes 6,

7 and 8). Phosphorimager analysis indicated that the amount of genome plus antigenome for

rHMPV+G1F23 compared to HMPV83 and rHMPV was 1.0 and 0.5, respectively (corrected for the

extra copy of the G gene present in rHMPV+G1F23), values very similar to that observed with the F

35   probe noted above. In comparison, the relative amount of G mRNA for rHMPV-G1F23 compared to

HMPV83 and rHMPV was 14-fold and 15-fold, respectively. This would correspond to a 14- to 30-

fold higher level of G mRNA for rHMPV+G1F23 compared to HMPV83 and rHMPV. Thus, the expression of the G and F genes by the rHMPV+G1F23 virus indeed was greatly increased.

    Yet another strategy for designing improved immunogenic compositions against HMPV is to modify the ORFs encoding protective antigens so that the codon usage is consistent with efficient

5   translation. Specifically, for many of the codons in a given ORF, the degeneracy of the genetic code allows for more that one choice of codon without changing the amino acid coding assignment. In a number of cases, specific codon choices have been shown to be associated with efficient translation while, conversely, other codon choices are associated with decreased efficiency of translation. Thus, the ORFs encoding HMPV antigens can be re-engineered to contain codon choices consistent with

10   efficient translation. This can be done for ORFs in their natural genome positions as well as for ORFs that have been shifted or for heterologous ORFs that have been inserted.

    Another exemplary embodiment of the current disclosure involves using a single attenuated HMPV backbone to make immunogenic compositions against more than one HMPV strain, subgroup or serotype. In this application, the protective antigen genes of an attenuated derivative of HMPV,

15   such as strain 83, are replaced singly or in toto by the corresponding genes of a heterologous HMPV. Provided that most of the attenuating mutations of the attenuated parent HMPV lie in genes other than the protective antigen genes, this results in a chimeric virus in which the attenuated backbone of the parent bears the protective antigens of the heterologous strain. This chimeric virus can then be administered on its own, or can be combined with the original parent to make a two-virus bivalent

20   immunogenic composition against the two viruses. This process can be repeated for additional heterologous strains as necessary.

    Yet another exemplary embodiment of the current disclosure involves the addition of one or more transcription cassettes encoding one or more foreign ORFs, as exemplified in Example II by GFP. In this application, the foreign gene is placed under the control of HMPV transcription signals

25   and is inserted into the genome in a location that does not interfere with virus viability, such as an intergenic region or a gene noncoding region. In this way, a variety of foreign proteins can be expressed, such as ones encoding protective antigens of heterologous pathogens, or immunomodulatory molecules.

    In yet another exemplary embodiment, rHMPV-GFP was recombinantly modified to

30   incorporate a Phenylalanine to Leucine mutation at amino acid position 456 in the L protein, a mutation designated F456L. This particular mutation was suggested by amino acid sequence alignment as corresponding to a position that encodes attenuating mutations in other, heterologous mutant nonsegmented negative stranded RNA viruses. The sequence alignment in FIG. 26A shows a phenylalanine-521 to Leucine (F521L) substitution in the L protein specified by a mutation in the L

35   gene of an RSV cold-passage temperature-sensitive (cpts) mutant cpts530 (Juhasz et al., J. Virol. 71:5814-9, 1999). The wild type F521 residue in the RSV sequence is exactly conserved among other mononegaviruses examined and, in particular, is present in HMPV. It should be noted that the amino

acid position of the corresponding residue is not the same in each L protein, reflecting differences in

length as well as small deletions and insertions elsewhere in the various individual molecules. Thus,

the corresponding residue is F456 in HMPV. From this comparative mapping analysis, the F456

residue in HMPV is identified as a target for either an identical, conservative, or even non-

5   conservative amino acid substitution (for example, substitution of the F456 residue by a leucine, or

by a conservative or non-conservative amino acid as compared to leucine). In this particular Example,

the F456L mutation was introduced into rHMPV-GFP and was readily recovered in a recombinant

virus, designated rHMPV-GFP-F456L. As shown in FIG. 27, this mutant replicated *in vitro* with an

efficiency that was indistinguishable from that of its direct parent, rHMPV-GFP, a characteristic that

10   is important for efficient viral recovery, evaluation and manufacture. Thus, this mutant is an excellent

candidate for further evaluation to determine its phenotypic characteristics *in vitro* and *in vivo*.

Further description of this general strategy, including discussion of FIGS. 26B-26F and 28, is

provided above, along with other strategies for the expeditious design of attenuated derivatives based

on the methods of this current disclosure.

15   Yet another strategy for making live attenuated vaccine candidates is the "Jennerian"

approach, which involves the use of a mammalian or avian virus to immunize against an antigenically

related human virus. This approach is named after Edward Jenner's successful use of cowpox as the

initial vaccine against smallpox in humans. This approach is based on the idea that a virus that has

evolved to replicate efficiently in its natural animal host often will replicate inefficiently and thus be

20   attenuated in the non-natural human host, reflecting a host range restriction. Such a host range

restriction typically is a very stable phenotype, being the aggregate effect of the many nucleotide and

amino acid sequence differences between the animal and human viral counterpart. A phenotype that

is based on many loci would be refractory to drift.

                Other examples of "Jennerian" vaccines include the use of bovine rotavirus or bovine PIV as

25   candidate vaccines against the respective human viruses. This can involve using the animal virus as it

is provided by nature, as illustrated by the use of bovine PIV directly as a vaccine (Karron *et al.*,

Pediatr Infect Dis J 15:650-654, 1996). Alternatively, the virus can be modified so that some of the

genes are derived from the animal virus and some from the human virus. In such a case, it is

preferable to have the major protective antigens be derived from the human virus, such that the

30   immunity that is induced will be maximally effective against the human virus, and one or more other

genes be derived from the animal virus such that they confer host range restriction. One such

situation involves chimeras between human and bovine parainfluenza type3 viruses: in one case, the

attenuated bovine backbone was modified so that the protective HN and F antigen genes were

replaced by those of the human virus, thus combining the attenuated bovine viral backbone with the

35   antigenic determinants of the human virus (Schmidt *et al.*, J. Virol. 74: 8922-8929, 2000), and in a

second case the human virus was attenuated by replacing a single gene, that of the nucleocapsid N

protein, with that of its bovine viral counterpart (Bailly *et al.*, J. Virol. 74:3188-3195, 2000).

Surprisingly, each of these bovine/human chimeric viruses was satisfactorily attenuated and immunogenic in rodent and nonhuman primates, and both are being prepared for clinical trials.

FIG. 30A illustrates chimeric HMPV vaccine candidates that were constructed by individually replacing the N, P or M ORFs of HMPV with the corresponding ORFs from APV

5   subtype C. At the amino acid level, the N, P and M genes of HMPV and APV-C are 90, 69, and 89% identical, respectively. For comparison, the N protein of bovine parainfluenza type 3 virus, whose substitution into the human PIV backbone provided a promising vaccine candidate, also was within this range, being 85% identical with its human viral counterpart. Furthermore, there are A, B and D subtypes of APV that are less closely related to HMPV, providing a source of additional replacements

10   ORFs whose greater degree of divergence might provide a higher degree of attenuation.

FIG. 30 Panels B, C and D illustrate the construction of chimeric N (Part B), P (Part C), and M (Part D) genes. In each panel, the upper sequence is that of a cDNA containing the native N, P or M HMPV gene, and the bottom sequence of each panel is a cDNA in which the HMPV ORF has been replaced by its APV counterpart. Both the native HMPV and chimeric HMPV/APV cDNAs

15   have been constructed to be flanked by restriction recognition sites for enzymes (*Bbs*1, *Bsm*B1, *Bfu*A1, as indicated) that cleave on the inner side of each recognition sequence to yield 4-nt overhangs. Since the actual cut site of these particular enzymes are not sequence specific, the cut sites can be designed to have the native HMPV sequence. This makes it possible to ligate the N, P and M genes together in a single step, as in FIG. 30E. Any combination of HMPV and HMPV/APV genes can be ligated. For

20   example, ligation of HMPV/APV N with HMPV P and HMPV M would yield a virus in which the N ORF alone was derived from APV. Single, double, or triple ORF replacements can readily be made. This represents one strategy for introducing attenuating host range mutations, namely a strategy in which the backbone is derived from HMPV and one or more individual ORFs from APV are introduced. An alternative strategy that also can be achieved by the methods of this current disclosure

25   involves starting with a full length APV genome and replacing the putative protective antigen genes, namely G, F and SH, by their HMPV counterparts in whatever combination is desired. This would combine the antigenic determinants of HMPV with the attenuated backbone of APV.

Example 4

30   **Analysis of Genetic Diversity Between Distinct Antigenic Subgroups of HMPV for Development
of Improved Immunogenic Compositions**

When van den Hoogen *et al.* first described the isolation of HMPV, they proposed that there was significant diversity among the various isolates (also called strains) and noted that "it is tempting to speculate that these subgroups of hMPV isolates represent different serotypes of hMPV" (Nat.

35   Med. 7:719-24, 2001). "Serotype" means that there would be insignificant antigenic cross-reactivity between HMPV subgroups following a primary immunization, and would necessitate the development of a separate vaccine for each. Even if the HMPV subgroups do exhibit antigenic cross

reactivity, it could be that sufficient antigenic diversity exists such that neutralization and protection across subgroups is significantly reduced compared to within a subgroup, and it might be necessary to have both such subgroups included in an effective immunogenic composition against HMPV.

Sequence diversity also was reported by Peret et al., albeit following analysis of a small segment of the genome, who proposed that there may be two "major groups or lineages" of HMPV (J. Infect. Dis. 185:1660-3, 2002). This also is supported by recent sequence analysis of the N, P, M and F genes of a number of HMPV isolates (Bastien et al., Virus Res. 93:51-62, 2003). The extent of sequence and antigenic diversity among circulating HMPV strains is of considerable importance to epidemiology, viral detection, and development of immunogenic compositions. Strains 83 and 00-1 as described herein are members of one of the proposed subgroups, and therefore a third strain was analyzed (75) that represents the second subgroup. Comparison of the three strains provided a measure of the degree of genetic diversity between subgroups (strain 83 or 00-1 versus 75) or within a subgroup (strain 83 versus 75).

A complete consensus sequence (FIGS. 39A-39D) was determined for the genome of strain 75 using the general procedures described in Example I above for strain 83. In this analysis, the sequence of the genomic termni of strain 75 were determined by 3´ and 5´ RACE as described in Example, I. Whereas strain 83 had yielded an unambiguous sequence for its entirety, strain 75 yielded a sequence in which a number of positions in the SH and G genes contained a mixture of two assignments. This suggested that the isolate was a mixture of two or more viruses that likely reflected quasispecies variants, either present in the original clinical sample or arising during in vitro passage. Analysis of cloned RT-PCR products spanning the SH and G genes produced two closely related sets of sequence that likely represented two different viruses. To obtain a sequence that could be unambiguously attributed to a single replication-competent HMPV 75 virus, the isolate was subjected to biological cloning by plaque isolation. Eight independent plaque-purified preparations yielded identical consensus sequences in the region of SH and G that was consistent with that of one of the two groups of cloned cDNA sequences. This sequence appears to represent the major constituent and was taken to be HMPV 75.

The genome of strain 75 is 13,280 nucleotides in length, compared to 13,335 for strain 83 and an estimated length of 13,378 for strain 00-1. The gene maps of strains 83 and 75, showing the cross-subgroup comparison, are shown in FIG. 29. Overall, the two genomes shared 80% nucleotide identity, compared to 81% between the A2 and B1 strains of RSV representing the two RSV antigenic subgroups A and B, respectively (Genbank accession numbers M74568 and AF013254, respectively). Similarly, the nucleotide sequence of isolate 75 (FIG.s 39A-39D) was 80% identical to that of isolate 00-1 (excluding the regions for which sequence was unavailable for 00-1), representing a second cross-subgroup comparison. The sequences of isolate 83 (FIG.s 37A-37D) and 00-1, representing an intra-subgroup comparison, were 92% identical. As will be detailed further herein, the level of divergence between the two HMPV subgroups bears considerable similarity to that

between the two antigenic subgroups of RSV. This finding was unpredicted and is of considerable value because there is extensive information on the significance of RSV diversity for epidemiology and development of immunogenic compositions, and this provides a very useful context for consideration of the divergence between HMPV subgroups, particularly since the overall magnitude

5   is similar. As will be noted herein, there were some differences between the diversity of HMPV versus RSV, but even here RSV provides a useful benchmark for evaluating these differences.

For most genera of the Paramyxovirus family, there is a "rule of six" whereby the nucleotide length of each genome is an even multiple of six (Kolakofsky et al., J. Virol. 72:891-899, 1998). In many cases, the placement of cis-acting signals relative to hexamer spacing also follows a conserved

10   pattern. However, HMPV replication and gene expression do not appear to be ruled by a phasing requirement based on the following observations: (i) the sequences for the three available isolates differ in length, as mentioned above; (ii) no integer from 2 to 9 is evenly divisible into each of the three genomes, and (iii) there was no apparent pattern of phasing for the gene boundaries within or between isolates. The absence of a phasing requirement also is suggested by the ability to recover a

15   number of recombinant viral mutants possessing a wide variety of lengths, as shown in previous Examples. The absence of such a requirement was unpredicted, and will greatly facilitate the construction of recombinant HMPVs.

The availability of complete genome sequences representing the two HMPV genetic subgroups provides an opportunity to examine the relatedness of the proposed HMPV transcription

20   signals between subgroups. This is illustrated in FIG. 31, which compares the putative GS, GE and intergenic sequences between the two subgroups. The GS and GE signals exhibited extensive sequence identity among the difference genes and between the two subgroups, and a number of residues were exactly conserved in all sequences. In contrast, the intergenic regions exhibited only 48% identity between subgroups, and non-coding gene regions that were not part of the putative GS

25   and GE signal exhibited only 54% identity. This sharp distinction in the pattern of sequence identity, combined with the functional analysis for transcription activity illustrated by the the GFP expression cassette in Example II above, provides guidance and an experimental method for a clear delineation of these important cis-acting sequences. Conversely, the identification of regions such as the intergenic regions and the non-translated, non-signal gene regions points out areas where the likelihood

30   is increased that modifications involved in gene insertions, gene rearrangements and other genetic engineering would be tolerated, providing guidance that will facilitate production of recombinant viruses for use in immunogenic compositions.

The conserved sequence at the up-stream end of each gene, which would include the GS signal, consists of 16 nucleotides, consensus: GGGACAAnTnnnAATG (FIG. 31). One unusual

35   feature of the GS signal of all HMPV isolates sequenced to date is the presence of ATG at positions 14-16 (FIGS. 8 and 31), which initiates the major ORF. In the SH gene of strains 75 and 00-1, there is an additional upstream ATG at GS positions 8-10: however, this start site is unlikely to be used

because of its unfavorable sequence context. The methods of the current disclosure allow for a
determination to be made whether the ATG at positions 14-16 is a functional element of the HMPV
GS signal or whether the conserved spacing of this translational start site plays some other role in
gene expression. This can readily be accomplished using either complete recombinant virus or the

5   minigenome system. The downstream end of each HMPV gene is delineated by the putative 12- to
15-nucleotide GE motif (consensus: AGTTAnnnAAAAA), consisting of (in positive sense) a highly
conserved AGTTA pentamer, followed by a poorly conserved A/T-rich trinucleotide followed by a
tract of 4 to 7 A residues. A sequence that resembles the GE motif also is present near the
downstream end of the HMPV leader region (FIG. 31), and the methods of the current disclosure will

10 clarify whether this functions like a GE signal.

        FIG. 32 shows the percent nucleotide identity and amino acid sequence identity between the
two HMPV subgroups (strain 83 versus 75) and within a subgroup (strain 83 versus 00-1) for the
various individual ORFs and their encoded proteins. With regard to nucleotide sequence identity
between the two subgroups, the HMPV ORFS shared 81-87% nucleotide identity except for SH and

15 G, which were substantially more divergent (69% and 59% identity, respectively). This pattern
resembled that of the two subgroups of HRSV, for which the percent nucleotide identity for the
various ORFs was 81-85% identical except for the more divergent M2-2, SH and G ORFs (69%,
77%, and 67% identity, respectively; FIG. 32). As noted above, the intergenic regions and the
noncoding gene regions (exclusive of GS and GE signals) were 48% and 54% identical, respectively,

20 between subgroups, compared to values of 42% and approximately 50%, respectively, for RSV
(Johnson et al., J. Gen. Virol. 69:2901-2906, 1988). Thus, the coding sequence for HMPV G was
only marginally more conserved than HMPV noncoding gene sequence in general (59% versus 54%).
Extensive nucleotide sequence divergence for the ORFs encoding the SH protein and, in particular,
the G protein also was observed between two strains of the same subgroup (strain 83 versus 00-1,

25 FIG. 32).

        FIG. 32 also shows the amino acid sequence relatedness of the various HMPV proteins
between the two subgroups (strain 83 versus strain 75), and within a subgroup (strain 83 versus 00-1).
With regard to the total proteome, the HMPV subgroups shared 90% amino acid sequence identity,
compared to 88% identity between the RSV subgroups. With regard to individual proteins, the most

30 conserved HMPV proteins were N, M, F, M2-1, and L (≥94% identity between subgroups), followed
by P and M2-2 (85%-89%), followed by the divergent SH (59%) and G (37%) proteins. SH and G
are the only ones for which the percent relatedness at the amino acid level was less than at the
nucleotide level, suggesting that amino acid substitutions were preferentially retained.

        The pattern of HMPV sequence diversity between subgroups has general similarities to that

35 of RSV (FIG. 32). For RSV, the "internal" virion proteins and the F protein constituted a group of
highly conserved species, while the M2-2, SH and G proteins were more divergent. Three
differences between HMPV and RSV were particularly noteworthy: (i) the HMPV M2-2 protein was

markedly more conserved (89% amino acid identity between subgroups) than its RSV counterpart (61%); (ii) the HMPV F protein also was more conserved than was the case for RSV (95% identity between subgroups versus 89%); and (iii) the HMPV SH and G proteins were markedly more divergent than their RSV counterparts (59% and 37% identity between subgroups, respectively,

5   compared to 72% and 55%, respectively). FIG. 32 also compares the HMPV proteins within a subgroup (strain 83 versus 00-1). For most of the proteins, the percent amino acid identity was very high (≤95%), with the SH and G proteins being more divergent (85% and 70% identity, respectively). The value of 70% sequence identity for G within an HMPV subgroup is much lower that the value of 91% reported for RSV G from the analysis of seven subgroup B strains (Sullender *et al.*, J. Virol.

10   65:5425-5434, 1991). Thus, the HMPV SH protein and, in particular, the G protein were markedly more divergent within and between subgroups than was the case for their HRSV counterparts. This finding was unpredicted and is important because it indicates these antigens would be ones for which it will be important to have both subgroups represented in an immunogenic composition once they are identified as significant protective antigens. A test for evaluating whether or not an HMPV

15   protein is a protective antigen is presented below.

It was also surprising to find that the HMPV M2-2 ORF and protein exhibited a high level of identity between the two putative subgroups, since M2-2 of RSV is poorly conserved (FIG. 32). In the case of RSV, the M2-2 protein was identified as a functional gene product by the observation that disruption of its ORF in infectious recombinant virus resulted in altered virus growth and RNA

20   synthesis (Bermingham and Collins, Proc. Natl. Acad. Sci. USA 96:11259-11264, 1999; Jin *et al.*, J. Virol. 74:74-78, 2000). As noted above, rHMPV in which the M2-2 ORF has rendered nonfunctional remains viable *in vitro*, although its phenotype remains to be fully defined. The high level of sequence conservation for the M2-2 ORF between HMPV subgroups suggests that it encodes a significant product, the absence of which might prove to be attenuating and useful in immunogenic

25   compositions. The RSV M2-2 ORF is expressed by a ribosomal stop-restart mechanism, whereby ribosomes that exit the M2-1 ORF apparently double back and initiate translation at the overlapping M2-2 ORF (Ahmadian *et al.*, EMBO J. 19:2681-2689). For the HMPV M2-2 ORF, there are two possible methionyl translational start sites located 38 and 50 nucleotides upstream of the translational termination codon of the M2-1 ORF. These two sites are exactly conserved among all three strains

30   compared here, and are embedded in highly conserved sequence. This is another example where the availability of the cross-subgroup comparison is useful for identifying structural features that are of potential significance, and whose possible functional roles can be tested using complete recombinant virus or minigenomes by the methods of this current disclosure.

The HMPV F, SH and G proteins appear to correspond to the three RSV surface

35   glycoproteins F, SH and G, although there was clear sequence relatedness between HMPV and HRSV counterparts only for F (33% amino acid identity). For RSV, G and F are the only significant neutralization antigens and are the major protective antigens. The HMPV F protein is identified as a

neutralization and protective antigen of HMPV in an Example below. The status of the remaining
HMPV proteins as potential neutralization and protective antigens can be determined by the same
method. The RSV F protein has a high degree of antigenic relatedness between subgroups (Johnson
et al., J. Virol. 61:3163-3166), consistent with its sequence relatedness (FIG. 32), and is the major

5   contributor to HRSV cross-subgroup neutralization and protection. For HMPV F, amino acid
sequence identity between subgroups was even higher than for RSV, and it is reasonable to anticipate
that this similarly will make a substantial contribution to cross-neutralization and cross-protection
between the HMPV subgroups. Conversely, the high degree of divergence of the G protein between
and within HMPV subgroups likely will compromise its contribution to cross-neutralization and

10   cross-protection to an even greater degree than is the case for RSV.

       FIG. 33 shows amino acid sequence alignments between and within a subgroup for the SH
(FIG. 33A) and G (FIG. 33B) proteins. The HMPV SH protein, like its RSV counterpart, is predicted
to be a type II glycoprotein that is inserted in the plasma membrane by a hydrophobic signal/anchor
sequence located near its amino terminus (FIG. 33A, boxed sequence), with a cytoplasmic amino

15   terminus and an extracellular carboxy terminus. The SH proteins of strains 75, 83, and 00-1 varied
in length (177, 179, and 183 amino acids, respectively; FIG. 33A) and were considerably longer than
their 64- or 65-amino acid counterparts in RSV. The predicted extracellular domain has 2-4 motifs
for N-linked glycosylation, one of which is conserved in all three isolates, as well as 3 or 4 potential
sites for O-glycosylation (Hansen *et al.*, Glycoconj. J. 15:115-130, 1998) that are clustered within

20   residues 75-81 in all three isolates (FIG. 33A). In addition, the SH proteins of the three isolates
contain 9 or 10 cysteine residues, which are mostly in the extracellular domain, 9 of which are
conserved among all three strains. The differences in length and amino acid sequence between the
SH proteins of the different isolates were concentrated in the extracellular domain.

       The HMPV G protein (FIG. 33B) also is a type II surface protein, bearing a general
25   resemblance (but no significant sequence relatedness) to the G protein of RSV. The amino acid
lengths of the G proteins of strains 75, 83, and 00-1 were 236, 219, and 236, considerably shorter
than their 289- to 299-amino acid counterparts in RSV. The HMPV G protein, like its RSV
counterpart, contains a high percentage of serine plus threonine residues (32- 35% for the three
isolates, compared to a data base average of 13%) and a somewhat elevated level of proline residues

30   (7-8.5%, compared to an average of 5%). Strains 83, -75 and 00-1 had 1, 4 and 4, respectively,
potential acceptor sites for N-linked glycosylation in the ectodomain. Each HMPV G protein
contained more than 40 predicted acceptor sites for O-linked carbohydrate (not shown): by analogy to
RSV it is likely that not all of these are used, and usage might be heterogeneous. The serine,
threonine, proline residues and the predicted sites for O-linked sugars were concentrated in the

35   predicted extracellular domain, suggesting that this region has a long, extended, heavily glycosylated
"mucin-like" structure, as is thought to be the case for RSV.

The HMPV G protein also resembles that of RSV in having most of the amino acid
divergence localized to the extracellular domain (FIG. 33B). Specifically, the cytoplasmic and
transmembrane domains combined were 64% and 96% identical between and within subgroups,
respectively, compared to the remarkably low values for the extracellular domain of 25% and 61%

5   identity between and within subgroups. For comparison, the value of 25% amino acid sequence
identity is comparable to that observed for the attachment hemagglutinin-neuraminidase protein of
PIVs from different genera, such as Genus Paramyxovirus versus Genus Rubulavirus or Avulavirus,
and is substantially lower than the value of 42-66% identity observed for the influenza A
hemagglutinin glycoprotein between subtypes H1, H2 and H3.

10 The HMPV G protein lacks several of the prominent features of its RSV counterpart. HMPV
G lacks the conserved 13-amino acid domain in the RSV G extracellular domain that is exactly
conserved among all RSV strains and is partially conserved in bovine RSV (Johnson et al., Proc.
Natl. Acad. Sci. USA 84:5625-5629, 1987; Teng and Collins, J. Virol. 76:6164-6171, 2002). Indeed,
the HMPV G ectodomain did not contain more than 3 adjacent amino acids conserved among all

15 three isolates. HMPV G also lacks the four conserved, closely-spaced cysteine residues in HRSV G
that partially overlap the 13-amino acid conserved domain, form a cystine noose, and include a CX3C
chemokine motif (Tripp et al., Nat. Immunol. 2:732-738 2001). The three HMPV G proteins in FIG.
33B each contained only 1 or 2 cysteine residues, with the only conserved one being at the inner face
of the cytoplasmic domain, representing a potential acceptor site for fatty acid acylation. HMPV G

20 also lacks a counterpart to the second methionyl translational start codon in the HRSV G ORF, which
gives rise to a secreted form and is conserved between HRSV subgroups (Roberts et al. J. Virol.
68:4538-4546, 1994). Only isolate CAN75 has an appropriately located potential counterpart
(methionine-43), and it is the fourth rather than the second methionyl residue. Thus, comparison of
sequence features whose presence or absence is consistent across subgroups helps guide the

25 identification of structural features as possible targets for mutational analysis and construction of
chimeric recombinant viruses for use in immunogenic compositions according to the methods of this
current disclosure.

The high level of divergence in HMPV SH and G proteins is consistent with the idea that
there is a preference for retaining amino acid changes in these proteins. It is particularly noteworthy

30 that the divergence was concentrated in the extracellular domain of each protein (FIG. 33A, B). A
codon-by-codon examiniation of the nucleotide coding sequences for the extracellular domains of F,
G and SH ORFs indicated that 75% of the codons containing a single nucleotide change were
associated with amino acid substitutions, compared to 50% for SH and 7% for F. Based on the
findings herein, it is suggested that the extraordinary frequency of amino acid substitution per

35 nucleotide substitution in HMPV G (and to a lesser extent SH) reflects two factors: (i) selective
pressure for amino acid change, which might come from host immunity, and (ii) the ability of the
protein to tolerate substitution, which might be due to its proposed extended, unfolded nature. In

-93-

contrast, HMPV F appears to resemble HRSV F, which is one of the more highly conserved proteins despite its status as a major HRSV neutralization and protective antigen. This likely reflects functional and structural constraints on amino acid substitution in this folded, globular glycoprotein. The time scale of nucleotide and amino acid substitution for HMPV is not known, but it was noted

5   that, for HRSV, it is difficult to detect antigenic drift and thus the differences probably reflect a slow accumulation over years. These findings indicate that diversity in SH and G is an important consideration for HMPV epidemiology, virus detection, and development of effective immunogenic compositions.

In conclusion, the present Example defines the amount genetic diversity that exists between

10   the two proposed HMPV genetic subgroups and shows that, overall, the diversity between subgroups is similar in magnitude to that observed between the well-characterized RSV antigenic subgroups, although certain specific differences were observed. By comparing the two divergent HMPV subgroups, the identification of transcription signal motifs was further substantiated. Conversely, the identification of poorly-conserved regions such as the non-signal, non-protein-coding gene regions

15   and the intergenic regions provides guidance in selecting these sites as being likely to be amenable to genetic manipulation. Importantly, the F glycoprotein was shown to be highly conserved between the two subgroups, which highlights the usefulness of this surface protein for incorporation in recombinant HMPV and chimeric recombinant HMPV of the current disclosure for production of broadly cross-reactive immunogenic compositions. Conversely, the other two viral surface proteins,

20   SH and G, were demonstrated to be highly divergent between the subgroups, and will therefore be employed within recombinant HMPV and chimeric recombinant HMPV to contribute to subgroup-specific, rather than cross-subgroup, immunity (although they will often be used in multivalent immunogenic compositions as described herein above). The diversity of the two genetic subgroups at the antigenic level is characterized herein, and the importance of the F protein as a major

25   neutralization and protective antigen is demonstrated.

Example 5

The Use of Experimental Animals to Evaluate HMPV Infectivity and Immunogenicity

Generation of Hamster Polyclonal Anti-HMPV Antibodies to the 75 and 83 Strains

30   To demonstrate that HMPV is infectious in a rodent animal model and can elicit an immune response characterized by production of HMPV-specific antibodies in the model host, three groups of 8- to 10-week-old golden Syrian hamsters (*Mesocricetus auratus*) were infected intranasally (IN) with approximately 10^3-10^4 $TCID_{50}$ of: a mixture of HMPV strains 75 and 83, HMPV 75 alone, or HMPV 83 alone. As described in the previous Example, strains 75 and 83 (also referred to as

35   CAN75 and CAN83, respectively) represent the two genetic subgroups of HMPV and thus provide an evaluation of the biological characteristics of viruses from each subgroup and, in particular, give a measurement of the antigenic diversity between subgroups. Serum samples were collected on day 28

or 44 post-immunization and examined for their ability to detect HMPV infected monkey kidney cells (LLC-MK2) by indirect immunofluorescence. LLC-MK2 cells grown in OptiMEM (Invitrogen GIBCO) on glass slides were infected with a mixture of HMPV strains 75 and 83, strain 75 alone, or strain 83 alone, and were incubated at 32°C in OptiMEM medium with 5 µg/ml trypsin. At

5 approximately 70 hours post-infection the cells were fixed and permeabilized with 3% formaldehyde and 0.1% Triton-X100 in PBS, as described previously (Skiadopoulos and McBride, J. Virol. 70:1117-24, 1996). Indirect immunofluorescence was performed with pooled hamster sera from animals that had been inoculated with HMPV 75 + 83, and fluorescein (FITC)-conjugated goat anti-Syrian Hamster IgG antibodies (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) were

10 used for indirect immunofluorescence. As a control, serum from an adult human seropositive for HMPV was also used to detect HMPV infected cells in a comparable indirect immunofluorescence assay. Immunofluorescence was detected and images were collected on a Leica TCS-SP2 (Leica Microsystems, Mannheim, Germany). Images were processed using the Leica TCS-NT/SP software (version 1.6.587), Imaris 3.1.1 (Bitplane AG, Zurich Switzerland), and Adobe Photoshop 5.5 (Adobe

15 systems). Both the human serum and the serum from hamsters infected with HMPV strains 75 and 83 were able to detect LLC-MK2 cells infected with either HMPV strain 75 or 83, demonstrating that IN- administered HMPV is infectious and immunogenic in hamsters. Sera from animals that received strain 75 alone or strain 83 alone were further analyzed as described herein.

20 *Generation of Rabbit Polyclonal Antibodies to HMPV Strains 75 and 83*

Specific pathogen free rabbits (Harlan Sprague Dawley) were surgically implanted with sterilized plastic wiffle balls and were immunized by injection of approximately 10^5 TCID$_{50}$ of HMPV strain 75 or 83 into the wiffle ball, as described previously (Clemons *et al.*, Lab. Anim. Sci. 42:307-11, 1992). Fluid present in the chambers was collected on day 29, and the animals received

25 two boosts by injection of HMPV preparations that had been purified and concentrated by centrifugation in sucrose gradient following conventional procedures: the first boost was on day 29 and the second was on day 57. Fluid present in the chambers was collected again on day 71. These sera were analyzed as described herein.

30 *Virus Titration and Serum Antibody Infectivity Neutralization Assays*

Viral titer was quantified by serial 10-fold dilutions of virus applied to LLC-MK2 monolayer cultures on 96-well plates that were then incubated for 7 days at 37°C in OptiMEM medium with 5 µg/ml added trypsin, as described previously (Newman *et al.*, Virus Genes 24:77-92, 2002). Virus infected monolayers were detected by incubation with hamster polyclonal antiserum,

35 which had been raised by IN infection with a mixture of strains 75 and 83 as described above, followed by a second antibody consisting of peroxidase-conjugated rabbit anti-Syrian hamster IgG.

Bound antibody-antigen complexes were detected by immunoperoxidase staining achieved with ECL chromogenic substrate.

The susceptibility of HMPV to neutralization by HMPV-specific antiserum was determined using an endpoint dilution neutralization assay performed with heat inactivated hamster, rabbit or

5   chimpanzee sera. The hamster and rabbit sera were prepared as described above. The chimpanzee sera were from 31 caged animals, as described further herein. Briefly, replicate aliquots each containing approximately 100-200 $TCID_{50}$ of HMPV 75 or 83 were mixed with aliquots from a dilution series prepared from a heat-inactivated serum sample and incubated at 37°C for 1 hr. The virus-antiserum mixtures were then transferred to LLC-MK2 monolayers in 96-well plates and the

10   cells were incubated for 1 hour at 32°C. The monolayers were washed two times with OptiMEM to remove serum and were overlaid with OptiMEM supplemented with 5μg/ml added trypsin. The cells were then incubated for 7 days at 32°C and infected monolayers were detected by immunoperoxidase staining with the polyclonal hamster anti-HMPV 75+83 antiserum and peroxidase-conjugated rabbit anti-Syrian hamster IgG and ECL chromogenic substrate, as described above. The neutralization titer

15   is read as the highest dilution of antibody at which the cultures were 50% reduced for infection. This assay was used to determine the HMPV neutralization titer of serum from infected animals, and also was used to screen animals to determine whether they had previously been exposed to HMPV.

With the availability of the HMPV-GFP virus, the neutralization assay can now be simplified. In this case, replicate aliquots of HMPV-GFP virus (approximately 100-200 $TCID_{50}$)

20   would be mixed with aliquots of a dilution series of serum as above, incubated for 1 hour at 32°C, and transferred to LLC-MK2 cells. The cells would be washed to remove serum, overlaid with OptiMEM supplemented with 5μg/ml added trypsin, and incubated for 7 days at 32°C. Infected cells could be visualized directly by fluorescent microscopy. The neutralization endpoint would be indicated by the appearance of green fluorescence, indicative of infection. Many of the details of this

25   assay can be varied as necessary, such as the period of incubation of HMPV-GFP and antibody, the choice of cell substrate, and the period of incubation of the cells. In particular, the use of HMP-GFP means that the cells can be monitored by fluorescent microscopy to select an optimal time at which to read the assay. The efficiency of virus titration was compared by visualization of GFP verus immunostaining and found to be identical. However, detection by GFP was easier because the

30   progression of the virus infection can be monitored and the plates read at an optimal time. In contrast, immunostaining can only be done once, and the timing is determined by estimating cytopathology by eye, which is more qualitative and not as precise. A further advantage is that GFP can be read directly, whereas immunostaining is a multi-step, time-consuming process requiring antibodies and development reagents. Furthermore, the washing steps need in immunostaining frequently result in

35   damage to or removal of the cell monolayer, which can complicate or ruin the assay. Thus, using rHMPV-GFP as an indicator virus for antibody titration (or evaluation of antiviral agents) provides a better method for titration.

Evaluation of the Antigenic Relatedness of Strains 83 and 75

As described in the previous Example, it has been suggested that HMPV isolates can be segregated into two genetic subgroups (van den Hoogen *et al.*, Nat. Med. 7:719-24; Peret *et al.*, J.

5   Infect. Dis. 185:1660-3, 2002). The previous Example described in detail the level of genetic diversity between the two proposed subgroups. The present Example (and also Example VI) describes the amount of antigenic difference between the two subgroups. The extent of antigenic diversity among circulating HMPV strains is of considerable importance to epidemiology and for development of effective immunogenic compositions.

10   The antigenic relatedness between strains 75 and 83 was investigated by a cross neutralization study. As described above, antiserum was raised in rabbits by parenteral immunization of gradient-purified HMPV 75 or 83 followed by a boost of the same material, with serum obtained following each step. In addition, as described above, hamsters were infected IN with strain 75 or 83 and sera were obtained. These individual sera were assayed for the ability to neutralize both strain 75

15   and 83. Thus, this would compare the ability of antibodies from an animal exposed to either strain to neutralize the infectivity of the homologous strain versus its ability to neutralize the heterologous strain. If extensive antigenic differences exit, each serum should be much less effective in neutralizing the heterologous strain compared to the homologous strain.

As shown in Table 1, hamsters were immunized by intranasal infection with strain 83 or 75

20   and the sera were collected on day 44 and evaluated for the ability to neutralize each strain *in vitro*. Of the eleven animals that were infected with strain 75, there was no consistent pattern in which the homologous 75 strain was neutralized more efficiently than the heterologous 83 strain: four animals neutralized the homologous strain more efficiently (up to 3.0 $\log_2$ difference, with animal 2164 exhibiting the greatest difference), but one (animal 2168) showed no difference and the remainder

25   neutralized the homologous strain less efficiently (up to 3.0 $\log_2$ difference, animal 2174). Conversely, all ten animals infected with strain 83 yielded sera that neutralized the homologous 83 strain more efficiently than the heterologous 75 strain, with a difference ranging from 0.4 $\log_2$ (animal 2203) to 3.4 $\log_2$ (animal 2201). Thus, each of the HMPV strains representing the two heterologous subgroups induced antibodies that efficiently neutralized the homologous and

30   heterologous viruses, although antibodies from animals infected with strain 83 appeared to be somewhat less effective in neutralizing the heterologous versus the homologous virus.

Also as shown in Table 1, rabbits were immunized with either strain of HMPV and then boosted with the same strain, and post-primary immunization and post-boost sera were collected and tested for the ability to neutralize the homologous and heterolgous subgroup virus. The initial

35   immunization of rabbits resulted in sera that had relatively low neutralizing titers against either strain of HMPV, with titers in the range of $\leq$4.3 $\log_2$ to 8.4 $\log_2$. With these primary sera, there was no consistent difference between the efficiency of neutralization of the homologous versus the

heterologous strain. Following the boost, the titers were substantially higher, in the range of 6.9 $\log_2$ to 11.4 $\log_2$, and in most cases the neutralization titer against the homologous strain was greater than against the heterologous strain. However, the difference was relatively modest, ranging from either no difference to a difference of 2.6 $\log_2$. For example, animal R030, which had been immunized and

5   boosted with strain 75, had a titer of 11.4 $\log_2$ against the homologous strain 75 and a titer of 9.9 $\log_2$ against the heterologous strain 83, corresponding to a 1.5 $\log_2$ difference. The other rabbit (R031) that was immunized and boosted with strain 75 yielded serum that neutralized the homologous strain 75 four times (2.0 $\log_2$) more efficiently than the heterologous strain 83. In the converse situation, with animals (R032 and R033) that had been immunized and boosted with strain 83, the titers following

10   the boost either showed no difference for the heterologous versus homologous strain (animal R03), or showed 0.5 $\log_2$ higher homologous versus heterologous titer (animal R033). Thus, out of the eight post-immunizaton and post-boost rabbit sera, one had a lower homologous versus heterologous titer, three had no difference, and four had a greater homologous versus heterologous titer, with the greatest difference being 2.6 $\log_2$. This indicates a trend towards greater homologous versus

15   heterologous reactivity, but the inconsistent nature of the pattern and the low magnitude is consistent with the interpretation that the two strain exhibit a very high degree of cross-neutralization *in vitro*. Thus, the high degree of antigenic relatedness observed with post-infection hamster sera was confirmed with rabbit sera both upon initial immunization and following a boost.

The high degree of antigenic cross-reactivity observed *in vitro* indicates that at least one

20   major HMPV neutralization antigen or major epitope is conserved between the HMPV subgroups. The genetic analysis presented in the previous Example showed that, of the three HMPV surface proteins F, SH and G, the F glycoprotein was highly conserved between subgroups. In contrast, the SH and G glycoproteins were highly variable and neither protein had no more than three contiguous conserved amino acid in the extracellular domain, suggesting that conserved antigenic sites would be

25   rare or nonexistent. The HMPV neutralization and protective antigens had yet been identified previously. In the case of RSV, the F and G surface proteins are the only significant neutralization antigens and the major protective antigens (Connors *et al.*, J. Virol. 65:1634-1637 1991), and in the case of the human PIVs the two surface proteins F and HN also are the neutralization and major protective antigens. A subsequent Example (Example VI) will illustrate identification of a major

30   HMPV neutralization and protective antigen effective in conferring protection against infection with either genetic subgroup.

Table 1: Homologous and heterologous HMPV antibody responses in rodents following immunization with HMPV strain CAN75 or CAN83

Rodent species and number	Immunizing virus[a,b]	Serum-neutralizing antibody titer[c] (recip. log_2) against:	
		CAN75	CAN83
Hamsters			
2163	CAN75	8.9	6.9
2164	CAN75	8.3	5.3
2165	CAN75	7.9	5.9
2169	CAN75	9.9	10.4
2170	CAN75	8.4	7.3
2166	CAN75	8.4	8.9
2167	CAN75	8.9	9.9
2168	CAN75	8.4	8.4
2173	CAN75	9.9	12.7
2174	CAN75	9.4	12.4
2175	CAN75	9.9	11.9
Hamsters			
2201	CAN83	7.3	10.7
2202	CAN83	8.4	11.3
2203	CAN83	10.9	11.3
2204	CAN83	7.9	10.3
2205	CAN83	7.9	10.3
2206	CAN83	8.9	11.7
2207	CAN83	7.9	10.3
2208	CAN83	8.9	11.7
2209	CAN83	9.9	12.3
2210	CAN83	8.9	10.3
Rabbits			
R030	CAN75	$\leq 4.3^d$	5.9^d
R030 (p.b.)	CAN75	11.4^e	9.9^e
R031	CAN75	$\leq 4.3^d$	$\leq 4.3^d$
R031 (p.b.)	CAN75	11.4^e	9.4^e
Rabbits			
R032	CAN83	$\leq 4.3^d$	6.9^d
R032 (p.b.)	CAN83	6.9^e	6.9^e
R033	CAN83	8.4^d	8.4^d
R033 (p.b.)	CAN83	8.4^e	8.9^e

a. Hamsters were inoculated intranasally with 10^4 $TCID_{50}$ of the indicated strain of HMPV.

b. Rabbits were immunized parenterally with 10^5 $TCID_{50}$ on day 0 with the indicated strain of HMPV, and were boosted on day 28 with 10^7 $TCID_{50}$ of gradient-purified virus of the same strain of HMPV.

c. Hamster neutralizing antibody titer, day 44 post-immunization serum. All animals had pre-immunization neutralizing antibody titers of ≤ 1.0.

d. Rabbit neutralizing antibody titer, day 28 post-immunization. All animals had pre-immunization neutralizing antibody titers of ≤ 1.0.

e. Rabbit neutralizing antibody titer post-boost, day 70 following the initial immunization. All animals had pre-immunization neutralizing antibody titers of ≤ 1.0.

p.b.: post-boost

Prevalence of HMPV Serum Antibodies in Chimpanzees

5   Thirty-one captive chimpanzees (*pan troglodytes*) were screened for the presence of serum

neutralizing antibodies to HMPV strain 75 or 83, using an *in vitro* infectivity neutralization assay

with HMPV strain 75 or strain 83 as the antigen, (Table 2).   Nineteen animals (61%) were found to

be seropositive for either one or both HMPV strains. Serum neutralizing antibody titers to HMPV

strain 75 ranged from undetectable ($\leq$ 1.0 log$_2$) to $\geq$ 9.6 log$_2$. Serum neutralizing antibody titers to

10   HMPV strain 83 ranged from $\leq$ 1.0 log$_2$ to 8.6 log$_2$. Twelve animals (39%) did not have detectable

HMPV-75 neutralizing titers: these 12 animals also were seronegative to strain 83. Four additional

animals were seropositive to strain 75 but seronegative to strain 83. These four animals had very low

neutralizing antibody titers (2.6 – 4.0 log$_2$), and their specificity is provisional. The prevalence of

HMPV antibodies in chimpanzees indicates that these animals can become readily infected with

15   HMPV in captivity, most likely from their human handlers. It also is possible, and indeed likely, that

there is animal-to-animal transmission. The ability of these animals to be infected by contact from

humans or other animals is a strong indication of permissiveness to HMPV. The finding that very

few animals were seropositive for only one of the two strains suggests that these strains are highly

related antigenically in primates and likely represent only a single HMPV serotype.

20

Table 2: Prevalence of HMPV-neutralizing antibodies in chimpanzee sera

Sample number	Chimpanzee ID No.	Serum neutralization titer (recip. log$_2$) to indicated HMPV strain[1]	
		75	83
1	99A005	6.6	4.6
2	A0A001	7.0	5.6
3	A0A002	5.6	4.0
4	A1A005	1.0	≤1.0
5	A1A007	≤1.0	≤1.0
6	A1A001	2.6	3.6
7	A1A002	5.6	3.6
8	A1A003	3.6	≤1.0
9	A1A006	≤1.0	≤1.0
10	A0A006	4.0	≤1.0
11	A0A007	4.6	3.6
12	A0A005	2.6	≤1.0
13	A0A009	3.6	≤1.0
14	AOA003	7.6	4.6
15	99A004	7.6	6.6
16	A2A001	≤1.0	≤1.0
17	A2A004	≤1.0	≤1.0
18	A2A006	≤1.0	≤1.0
19	A2A009	≤1.0	≤1.0
20	A2A011	6.0	4.6
21	A2A013	6.6	4.0
22	A2A014	6.6	5.6
23	98A008	8.6	6.0
24	99A001	8.0	5.0
25	99A002	8.0	6.6
26	99A003	9.6	8.6
27	A1A010	≤1.0	≤1.0
28	A1A012	≤1.0	≤1.0
29	A1A013	≤1.0	≤1.0
30	A2A007	≤1.0	≤1.0
31	A2A008	≤1.0	≤1.0
Control serum (pre)2		≤1.0	≤1.0
Control serum (post)3		8.3	8.3

1. Numbers in parentheses are the titers expressed as the reciprocal dilution log$_2$.
2. pre-immune serum from hamsters before inoculation with both HMPV 75 and 83.
3. Post-immunization serum obtained from hamsters following inoculation with both HMPV 75 and 83.

HMPV Infection of Chimpanzees and Monkeys: Virus Replication, Immunogenicity and Effect of
Previous Exposure to HMPV

The ability of HMPV strains 75 and 83 to infect chimpanzees identified as seropositive or

seronegative by the HMPV neutralization assay described above was evaluated. As shown in Table

5   3, eight animals were organized into four groups (two animals per group): group 1 was seronegative

for strain 75 and 83. Group 2 and group 4 were seropositive for both strains. Group 3 was

seronegative for 83 and had low or no detectable neutralization titers to strain 75. Groups 1 and 2

were administered strain 75, and groups 3 and 4 were administered strain 83. The animals were

inoculated intranasally (IN) and intratracheally (IT) with 1 ml per site of 1 X $10^{5.2}$ TCID$_{50}$/ml of the

10   indicated virus. Administration was by both the intranasal (IN) and intratracheal (IT) routes to

provide the opportunity for the virus to replicate at each site (Hall *et al.*, Virus Res. 22:173-84, 1992;

Crow *et al.*, Vaccine 12:783-790, 1994). Animals were housed and specimens collected as

previously described (Durbin *et al.*, J. Infect. Dis. 179:1345-51, 1999; Skiadopoulos *et al.*, Virology

260:125-35, 1999). The animals were observed clinically for 16 days post-inoculation.

15   Nasopharyngeal swabs were collected on days 1 to 10 and 12 post-infection and tracheal lavages

were collected on days 2, 4, 6, 8, 10, and 12 post-infection. Each of the four seronegative animals

(groups 1 and 3) had nasal discharge on days 7, 8, 9 and 10 post-infection. As a precautionary

measure, groups 1 and 3 were started on penicillin on day 8, because of evidence of congestion and

noise in the lungs. One of the animals in group 1 had thick mucus in the day 8 post-infection TL

20   sample and had decreased appetite. The seropositive animals (groups 2 and 4) showed no signs of

illness throughout the course of the study. Thus, HMPV seronegative chimpanzees can develop

illness following infection with the 75 and 83 HMPV strains demonstrating that these viruses are

infectious and virulent in chimpanzees. Since the recombinant HMPV strain 83 virus differs from its

biologically-derived parent by only four nucleotide differences involved in creating the *Nhe*I marker

25   in the M/F intergenic region (FIG. 7), it is also considered a "wild type" virus in terms of genomic

and phenotypic characteristics. Importantly, animals that were seropositive to both strains were

protected from disease following challenge with either strain. This appears to be the first observation

demonstrating the feasibility of immunizing against HMPV: since natural exposure protects against

disease, it is reasonable to expect immunization with an appropriate immunogenic composition

30   comprising a recombinant HMPV of the current disclosure will elicit a desired immune response as

well. These results indicate that serum neutralizing antibody titers of 4.6 (animal AOA003) to 7.0

(animal AOA001) log$_{10}$ are sufficient to confer protection from HMPV disease. Results obtained

with chimpanzees are particularly relevant given the close evolutionary relationship between

chimpanzees and humans.

Table 3: Infection of chimpanzees with HMPV

Group	Chimpanzee ID No.	Serum neutralization titer to HMPV strain:		HMPV administered[1]	clinical signs (day post-infection)	
		75	83		rhinorrhea	cough
1	A1A005	≤1.0	≤1.0	75	6,7,8,9,10,12	
	A1A007	≤1.0	≤1.0		7,8,9,10	
2	99A005	6.6	4.6	75		
	A0A001	7.0	5.6			
3	A1A006	≤1.0	≤1,0	83	7,8,9,10	
	A0A005	2.6	≤1.0		7,8,9,10	5
4	AOA003	7.6	4.6	83		
	99A004	7.6	6.6			

1. The animals were inoculated intranasally (IN) and intratracheally (IT) with 1 ml per site of $10^{5.2}$ $TCID_{50}$/ml of HMPV strain 75 or strain 83.

The virus titers in the nasopharyngeal swabs and tracheal lavages taken from the HMPV-infected chimpanzees described in Table 3 were determined by an end point dilution assay with viral infectivity detected by immunostaining. As shown in Table 4, the chimpanzees shed relatively low levels of virus. This was particularly noteworthy for the animals in group 1, which were seronegative for both strains, and group 3, which was seronegative for strain 83: animals even though all of the animals in these groups exhibited clinical disease signs in response to infection, the mean peak titers of virus recovered from the nasopharyngeal swabs ranged from 1.9 to 3.2 log_{10}, and the titers from the tracheal lavage were 1.8 to 2.0 log_{10}. The swabs and lavages from animals in groups 2 and 4, which were seropositive to HMPV, either lacked detectable HMPV or had a very low titer (1.5 log_{10}). Thus, the lack of disease signs correlated with a reduced or undetectable level of virus shedding. The apparent incongruity between the low level of HMPV shedding in the presence of disease symptoms might reflect difficulty in retrieving infectious virus from secretions, possibly due to inactivation by host immune factors or a high level of cell-association of HMPV in the epithelial cells lining the respiratory tract of the chimpanzees. Since the chimpanzee is so closely related to humans, this raises the possibility that detection of HMPV in human secretions might also be inefficient. The inability to detect substantial HMPV shedding was unexpected, since RSV is known to be labile and cell-associated *in vitro*, but nonetheless is shed from RSV infected chimpanzees presenting with RSV-associated illness at a level that is 100 to 1000-fold greater than was the case for HMPV-infected chimpanzees (Belshe *et al.*, J. Med. Virol. 1:157-162, 1977). Alternatively, the level of HMPV replication in chimpanzees may indeed be low, but infection may trigger immune- or chemokine-mediated pathology resulting in development of disease symptoms (Glass *et al.*, Curr. Opin. Allergy Clin. Immunol. 3:467-473).

Serum samples taken four to six weeks post infection contained high levels of serum
neutralizing antibodies for every experimental group, with no clear difference between animals that
had been seronegative or seropositive prior to the experimental infection. The post-immunization
titers ranged from 7.4 to 10.2 $\log_{10}$, considerably higher than the range of 4.6 to 7.0 $\log_{10}$ that was

5   associated with protection from clinical disease. Interesting, in this small sample there was no
evidence of antigenic differences between strains 75 and 83. Specifically, seronegative animals that
were immunized with either strain (groups 1 and 3) developed antibodies with titers that were
somewhat higher against strain 83 than against strain 83, irrespective of the immunizing virus.

The animals that were seronegative for the strain used in the primary infection were next

10   challenged with the heterologous HMPV strain. As shown in Table 4, both groups of animals were
completely protected from cross-challenge, indicating that previous infection by one HMPV lineage
protects chimpanzees from subsequent infection by the heterologous lineage. Thus, in the
experimental animal that most closely resembles the human, immunity induced by an HMPV strain
representing either genetic subgroup protected against infection with an HMPV strain from the

15   heterologous subgroup.

It also was of interest to investigate the replication and immunogenicity of HMPV 75 and 83
in monkeys, which are more plentiful, less expensive and easier to house than chimpanzees. Twenty-
two rhesus macaques and twenty African green monkeys were screened for serum neutralizing
antibodies to HMPV, and each animal was seronegative to HMPV ($\leq$1.0 $\log_2$). This limited sampling

20   suggested that these two species of monkeys are not readily infected with HMPV by exposure in
captivity. Four animals of each species were infected and intratracheally with $10^{5.2}$ TCID$_{50}$ of either
HMPV strain CAN75 or CAN83. Four animals of each species were infected and intratracheally with
$10^{5.2}$ TCID$_{50}$ of either HMPV strain CAN75 or CAN83 at each site, and nasopharyngeal swab
samples were taken at days 1 to 10 and 12 post-infection, and tracheal lavage samples were taken at

25   days 2, 4, 6, 8, 10 and 12 post-infection (Table 5). The samples were assayed for virus titer by end
point dilution. Illness did not develop in any of the monkeys. The infected rhesus monkeys had very
low titers of virus, ranging from 0.9 to 2.6 $\log_{10}$ TCID$_{50}$ (Table 5). Furthermore, the post-
immunization titers of serum neutralizing antibodies were also relatively low, ranging from 6.3 to 8.4
$\log_2$. Thus, rhesus monkeys support the replication of HMPV, but the level of replication is low.

30   Infected African green monkeys (Table 5) shed titers of HMPV ranging from 2.2 to 4.9 $\log_{10}$
TCID$_{50}$, and developed serum neutralizing antibody titers that ranged from 9.4 to 10.9 $\log_2$. The time
course of HMPV replication in samples collected from the nasopharynx and the trachea of the
African green monkeys is shown in FIG. 35, and indicates that virus shedding occurred over a broad
window of more than six days. These data showed that African green monkeys are permissive for

35   HMPV replication and, in particular, represent a suitable primate host in which to evaluate the
replication and immunogenicity of rHMPV derivatives for use in immunogenic compositions. In
particular, the highest titers of virus and antibody were obtained with strain 83, which is the one

represented by recombinant HMPV of this current disclosure. In this regard, the ability to monitor HMPV replication up to almost $5 \log_{10}$ with concomitant immunogenicity provides a wide window for assessing effects on replication and immunogenicity due to the incorporation of attenuating mutations.

5   Thirty days following the first infection, the African green monkeys were challenged with the heterologous HMPV strain (Table 5). Both groups of animals were completely protected from cross-challenge, indicating that previous infection by one HMPV lineage confers resistance to subsequent infection by the heterologous strain. These data indicate that African green monkeys represent a suitable primate host in which to evaluate the protective efficacy of HMPV immunogenic

10   compositions.

          The studies described above provide data on the primary antibody response to strain 75 or 83 in seronegative animals, and describe the ability of the resulting antibodies to neutralize the homologous versus heterologous subgroup virus. These data were analyzed statistically by the Archetti-Horsfall formula (Archetti and Horsfall, J. Exp. Med. 92:441-462, 1950) to determine the

15   degree of antigenic relatedness based on *in vitro* neutralization. Each species was calculated separately, showing that the percent relatedness of the two HMPV subgroups was 48%, 64% and 99%, respectively, based on the data from hamsters, Rhesus monkeys and African green monkeys, respectively. This compares with a value of 25% and 31% for the two subgroups of RSV, based on analysis of animal and human sera, respectively (Johnson *et al.* J. Virol. 61:3163-3166, 1987; Hendry

20   *et al.*, J. Infect. Dis. 157:640-647, 1988). These data indicate that the two HMPV genetic subgroups are highly related antigenically and do not represent distinct serotypes or, likely, not even significant antigenic subgroups. This high degree of relatedness for the two HMPV subgroups is based on *in vitro* neutralization, and thus needs to be taken with some caution, since cross-neutralization in vitro might be mediated by a single highly conserved antigen, whereas cross-protection in vivo might

25   require the contribution of more than a single antigen, or might be augmented by the inclusion of additional antigens. Nonetheless, the present findings provide the first indication that the genetic diversity of HMPV can readily be accommodated by an HMPV vaccine, and that such a vaccine might need to represent only a single HMPV strain.

TABLE 4: Level Of Replication, Immunogenicity And Cross-Protection Of HMPV Strains CAN75 And CAN83 In Chimpanzees

Group no.[a]	Virus administered	Serostatus at time of inoculation	Pre-immunization serum neutralization titer[d] to:		Level of virus replication[e] in:		Post-immunization (day 28) serum neutralization titer[d] to:		Level of challenge virus replication[f] in:	
			CAN75	CAN83	NP swab	TL	CAN75	CAN83	NP swab	TL
1	CAN75	negative[b]	$\leq 1.0 \pm 0.0$	$\leq 1.0 \pm 0.0$	1.9 ± 0.4	2.0 ± 0.8	8.7 ± 0.3	10.2 ± 0.3	$\leq 0.5 \pm 0.0$	$\leq 0.5 \pm$
2	CAN83	negative[b]	3.8 ± 1.5	$\leq 1.0 \pm 0.0$	3.2 ± 0.5	1.8 ± 0.3	8.9 ± 1.0	10.2 ± 0.3	$\leq 0.5 \pm 0.0$	$\leq 0.5 \pm 0.0$
3	CAN75	positive[c]	6.8 ± 0.2	5.1 ± 0.5	1.5 ± 0.0	$\leq 0.5 \pm 0.0$	7.4 ± 0.5	9.2 ± 0.2	nd	nd
4	CAN83	positive[c]	7.6 ± 0.0	5.6 ± 1.0	$\leq 0.5 \pm 0.0$	1.5 ± 0.3	8.4 ± 1.5	10.2 ± 0.2	nd	nd

a. Animals in groups of two were inoculated IN and intratracheally (IT) with $10^{5.2}$ TCID$_{50}$ of the indicated virus in a 1 ml inoculum at each site.

b. Chimpanzees in this group were seronegative (mean antibody titer of <1:4) for the homologous infecting HMPV strain at the start of the study.

c. Chimpanzees in this group were seropositive (mean antibody titer of > 1:20) for the homologous infecting HMPV strain at the start of the study.

d. Serum neutralization antibody titer to the indicated virus is expressed as the mean reciprocal log$_2$ ± standard error.

e. Level of virus replication is expressed as the mean of the peak virus titers (log$_{10}$ TCID$_{50}$/ml ± standard error) for the animals in each group irrespective of sampling day. The NP titers on day 0 were ≤0.5 log$_{10}$ TCID$_{50}$/ml. A value of ≤ 0.5 log$_{10}$ TCID$_{50}$/ml is assigned to samples with no detectable virus.

f. On day 35, animals were challenged IN and IT with$10^{5.2}$ TCID$_{50}$ of the heterologous strain of HMPV in a 1 ml inoculum at each site. Level of virus replication is expressed as the mean of the peak virus titers (log$_{10}$ TCID$_{50}$/ml ± standard error) for the animals in each group irrespective of sampling day. The NP titers on day 0 were ≤ 0.5 log$_{10}$ TCID$_{50}$/ml. The lower limit of detection is 1.0 log$_{10}$ TCID$_{50}$/ml.

nd: not done

TABLE 5: Level Of Replication, Immunogenicity And Cross-Protection Of HMPV Strains CAN75 And CAN83 In Two Species Of Monkeys

Primate species	No. of animals	Virus inoculum[a]	Level of virus replication[b] in:		Post-immunization (day 28) serum neutralization titer[c] to:		Percent antigenic relatedness[d]	Level of challenge virus replication[e] in:	
			NP swab	TL	CAN75	CAN83		NP swab	TL
Rhesus	4	CAN75	1.3 ± 0.1	0.9 ± 0.2	6.4 ± 0.6	7.2 ± 0.3	64	nd	nd
Rhesus	4	CAN83	1.6 ± 0.1	2.6 ± 0.3	6.3 ± 0.1	8.4 ± 0.3		nd	nd
African Green	4	CAN75	2.6 ± 0.5	3.2 ± 0.4	9.4 ± 0.3	10.8 ± 0.4	99	1.4 ± 0.1	0.8 ± 0.1
African Green	4	CAN83	2.2 ± 0.2	4.9 ± 0.1	9.5 ± 0.5	10.9 ± 0.4		0.6 ± 0.1	0.9 ± 0.2

a. Animals were inoculated IN and intratracheally (IT) with $10^{5.2}$ $TCID_{50}$ of the indicated virus in a 1 ml inoculum at each site.

b. The level of virus replication is expressed as the mean of the peak virus titers ($\log_{10}$ $TCID_{50}$/ml ± standard error) for the animals in each group irrespective of sampling day. Virus titrations were performed on LLC-MK2 cells. The lower limit of detection is 1.0 $\log_{10}$ $TCID_{50}$/ml. A value of $\leq$0.5 $\log_{10}$ $TCID_{50}$/ml is assigned to samples with no detectable virus.

c. Mean serum neutralization antibody titer to the indicated HMPV strain is expressed as the mean reciprocal $\log_2$ ± standard error. All animals were seronegative for both strains of HMPV at the start of the study (that is, day 0 reciprocal neutralization titer of $\leq$ 1.0 $\log_2$).

d. Percent antigenic relatedness was calculated using the formula of Archetti and Horsfall.

e. On day 30, animals were challenged IN and IT with $10^{5.2}$ $TCID_{50}$ of the heterologous virus in a 1 ml inoculum at each site. Level of virus replication is expressed as the mean of the peak virus titers ($\log_{10}$ $TCID_{50}$/ml ± standard error) for the animals in each group irrespective of sampling day. The lower limit of detection is 1.0 $\log_{10}$ $TCID_{50}$/ml.

nd: not done

Development of a small animal model of HMPV replication

The evaluation of HMPV and rHMPV derivatives is further facilitated by the availability of a small animal model for evaluating replication and immunogenicity *in vivo*. Hamsters were infected intranasally with 10^6 TCID$_{50}$ of either strain 83 or 75, and animals were sacrificed on days 3, 4, 5, 6 or 7 and the nasal turbinates and lungs were harvested and assayed for virus titer. As shown in Table 6, each virus replicated to a peak titer greater than 6 log$_{10}$ in the nasal turbinates and 3.6 to 4.4 log$_{10}$ in the lower respiratory tract. Thus, hamsters were permissive for both subgroup strains. As shown in Table 1 (and in Table 7), infection with strain 83 induced serum neutralizing antibody titers of 10.3 to 12.3 log$_{10}$ against the homologous strain, and infection with strain 75 induced titers of 7.9 to 9.9 log$_{10}$ against the homologous strain. The basis for the apparent lower immunogenicity of strain 75 is not known, although this finding will need to be confirmed in a further study in which virus replication and immunogenicity are measured for each strain in the same study. The present data show that hamsters are relatively permissive for HMPV replication and provide a convenient small animal model for evaluating HMPV replication and immunogenicity.

The hamster model was used to evaluate cross-protection between the two HMPV genetic subgroups, as had also been investigated in chimpanzees as shown in Table 4. Groups of hamsters were infected with either strain of HMPV or with L15 diluent as a negative control. After 6 weeks the hamsters were challenged with either strain of HMPV and the level of pulmonary challenge virus replication in the respiratory tract on day 4 was determined, as described above. As shown in Table 7, previous infection by either HMPV strain induced a high level of protection against the homologous and heterologous HMPV strain in the upper respiratory tract. The lower respiratory tract was completely protected from challenge with either strain. In addition, sera were collected 31 days following the first infection and assayed to determine the neutralizing titer against each strain (Table 7). The results were very similar to those shown previously in Table 1: infection with CAN83 induced antibodies that were modestly more effective in neutralizing the homologous versus heterologous strain, but antibodies induced by infection with CAN75 neutralized the homologous and heterologous strains with similar efficiencies. These cross-protection data and reciprocal-neutralization assays indicated that CAN83 and CAN75 are antigenically highly related.

TABLE 6: DAILY LEVEL OF REPLICATION OF HMPV STRAINS CAN75 AND CAN83 IN THE UPPER AND LOWER RESPIRATORY TRACT OF INFECTED HAMSTERS

Virus inoculum[a]	Tissue	Mean virus titer ($\log_{10}$ TCID$_{50}$/g ± S.E.) on day[b]:				
		3	4	5	6	7
HMPV-CAN83	Nasal turbinates	6.0 ± 0.5	5.7 ± 0.3	5.1 ± 0.3	3.7 ± 1.2	1.7 ± 0.5
	Lungs	3.6 ± 1.0	3.1 ± 0.3	3.0 ± 0.3	≤1.5 ± 0.0	≤1.5 ± 0.0
HMPV-CAN75	Nasal turbinates	6.6 ± 0.4	6.4 ± 0.4	6.4 ± 0.6	5.2 ± 0.5	4.2 ± 0.7
	Lungs	4.4 ± 0.6	4.3 ± 0.7	3.3 ± 0.2	≤1.5 ± 0.0	≤1.5 ± 0.0

[a]Hamsters in groups of 30 animals were inoculated intranasally with 10^6 TCID$_{50}$ of the indicated virus.

[b]Nasal turbinates and lung tissues from six animals from each group where harvested on days 3 through 7 post-infection. Virus present in tissue homogenates was quantified by serial dilution on LLC-MK2 monolayers at 32°C. Infected cultures were detected with hamster anti-HMPV antibodies and immunoperoxidase staining. Mean virus titer for each group of hamsters is expressed as $\log_{10}$ TCID$_{50}$/gram ± standard error. S.E.

5

TABLE 7: Hamsters Infected With HMPV Strain CAN83 Or CAN75 Are Protected Against Challenge With The Homologous And Heterologous HMPV Strains

Animals immunized with[a]	Mean serum-neutralizing antibody titer[b] (reciprocal log$_2$) against:		Mean virus titer (log$_{10}$ TCID$_{50}$/g) in nasal turbinates of hamsters administered the indicated challenge virus[c]:	
	CAN75	CAN83	CAN75	CAN83
CAN83	9.2 ± 0.2	10.4 ± 0.3	2.0 ± 0.2	1.7 ± 0.2
CAN75	10.9 ± 0.2	7.7 ± 0.2	≤1.5 ± 0.0	2.4 ± 0.5
L15 medium	≤1.0 ± 0.0	≤1.0 ± 0.0	5.3 ± 0.1	5.6 ± 0.2

a. Hamsters in groups of 12 were immunized by IN infection with $10^{5.5}$ TCID$_{50}$ of the indicated virus or mock-infected with L15 medium.

b. Sera were collected 2-3 days before and 31 days following the first infection, and the neutralizing titer against each of the two strains was determined. The pre-infection anti-HMPV serum titers were ≤1.0 (reciprocal log$_2$) for all animals in the study.

c. On day 42, six hamsters from each group were challenged IN with $10^{5.5}$ TCID$_{50}$ of CAN83 and the remaining six were challenged with CAN75. Nasal turbinates and lungs were harvested four days later, and the virus titer was determined on LLC-MK2 cells. The mean virus titer for each group of hamsters is expressed as log$_{10}$ TCID$_{50}$/gram of nasal turbinate ± standard error.

Example 6

Construction of a Human Parainfluenza Type 1 Virus (HPIV1) that Induces Neutralizing Antibodies and Protective Efficacy Against Both HMPV Genetic Subgroups

To examine the ability of the HMPV F protein alone to induce serum neutralizing antibodies and to confer protection against HMPV challenge, a recombinant HPIV1 expressing the CAN83 F protein (rHPIV1-F$_{83}$) was recovered in vitro from an HPIV1 antigenomic cDNA containing the CAN83 F protein ORF inserted upstream of the HPIV1 N gene and flanked by HPIV1 GS and GE transcription signals (FIG. 36A). Expression of the F protein was confirmed by indirect immunofluorescence of infected cells (FIG. 36 B). Thus, rHPIV1-F$_{83}$ would express all of the proteins of HPIV1 as well as the F protein of HMPV.

Groups of hamsters were immunized with CAN83, wild type rHPIV1, rHPIV1-F$_{83}$ or with L15 medium, and 33 days later sera were collected and tested for the ability to neutralize the CAN75 and CAN83 strains. As shown in Table 8, sera from animals immunized with CAN83 or rHPIV1-F$_{83}$ efficiently neutralized both strains of virus. In each case, neutralization of the homologous CAN83 strain was somewhat more efficient than for the heterologous CAN75 strain. The mean neutralization titer of sera from rHPIV1-F$_{83}$-infected animals was 1.2 log$_2$ lower than that observed from CAN83-infected animals, which corresponds well with the lower titer of rHPIV1 replication in hamsters in this experiment compared to CAN83. Thus, when adjusted for the level of virus replication, the rHPIV1- F$_{83}$ vector, expressing only a single CAN83 antigen, was as efficient in inducing CAN83-neutralizing antibodies as CAN83 itself.

Fifty days following immunization, the animals were challenged by intranasal infection with rHPIV1, CAN75 or CAN83. As shown in Table 8, animals previously infected with rHPIV1-F$_{83}$ had a 125-fold and 158-fold reduction in the upper respiratory tract in the level of replication of the CAN75 and CAN83 challenge virus, respectively. Animals were completely protected in the lower respiratory tract. The rHPIV1-F$_{83}$ virus also protected against HPIV1 challenge, indicating that this virus was able to protect against the three respiratory tract pathogens, namely CAN75, CAN83 and HPIV1. This method also can now be used to determine the relative contribution of each of the other HMPV proteins to the induction of neutralizing antibodies and resistance to challenge virus replication. Importantly, these findings identify the F protein of HMPV as a major neutralization and protective antigen. Furthermore, the F protein of the CAN83 strain induced neutralizing antibodies and protection that were effective against both CAN83 and CAN75. This highlights the importance of this specific antigen in developing vaccines effective against all strains of HMPV.

TABLE 8: A Recombinant HPIV1 Expressing The CAN83 F Protein (RHPIV1-F$_{83}$) Equally Protects Hamsters Against Challenge With HMPV Subgroup CAN75 And CAN83 As Well As Against HPIV1

Virus inoculum[a]	Mean serum neutralization titer (log$_2$) to indicated virus[b]:			Mean level of replication of the indicated challenge virus in the nasal turbinates[c]		
	rHPIV1	CAN75	CAN83	rHPIV1	CAN75	CAN83
L15 medium	≤1.0 ± 0.0	≤1.0 ± 0.0	≤1.0 ± 0.0	4.9 ± 0.3	5.0 ± 0.2	6.1 ± 0.3
CAN83	≤1.0 ± 0.0	8.3 ± 0.2	9.8 ± 0.2	4.6 ± 0.1	1.5 ± 0.0	1.5 ± 0.0
rHPIV1wt	6.1 ± 0.3	≤1.0 ± 0.0	≤1.0 ± 0.0	2.0 ± 0.2	4.4 ± 0.2[d]	5.8 ± 0.2[e]
rHPIV1-F$_{83}$	4.7 ± 0.2	6.0 ± 0.3	8.6 ± 0.2	1.9 ± 0.2	2.9 ± 0.3[d]	3.9 ± 0.1[e]

a. Groups of 18 hamsters were infected IN with 10^5 TCID$_{50}$ of the indicated virus or with L15 medium as a negative control.

b. Thirty-three days following the first infection, sera were collected and assayed to determine the neutralization titers against HPIV1, CAN75 and CAN83. 50 days following the first infection, six hamsters from each group were challenged IN with 10^5 TCID$_{50}$ of the indicated virus and the nasal turbinates were harvested after four days.

c. Virus present in the tissue homogenates was quantified and is expressed as the mean log$_{10}$ TCID$_{50}$/g for each group ± standard error.

d. Statistically significant difference between indicated values; $p < 0.01$ (unpaired t-test).

e. Statistically significant difference between indicated values; $p < 0.0001$ (unpaired t-test)

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Example 7

Construction of HMPV Recombinant Virus Expressing Protective Antigens for Both Putative Antigenic Subgroups of HMPV

As described above, different strains of HMPV can exhibit genetic diversity, especially in
5   the SH and G proteins, and there appear to be two major genetic subgroups. The preceding Example
identified the HMPV F protein as a major neutralization and protective antigen effective against both
subgroups. Thus, it is a preferred antigen for including in an HMPV vaccine. The preceding
Examples showed that it is possible to achieve a high degree of protective immunity against both
genetic subgroups by infection with a single HMPV from either subgroup. Furthermore, the F protein
10   was identified as a major neutralization and protective antigen effective against both subgroups.
However, it may be that optimal immunogenicity and protective efficacy will be achieved by
including the SH or G protein from either or both subgroups, or by including F protein from both
subgroups. In addition, the available information indicates that HMPV is a significant cause of
serious respiratory tract disease early in life, approximately coincident with RSV and the PIVs. Thus,
15   an immunogenic composition against HMPV likely would be administered at approximately the same
time in life (optionally in a combinatorial formulation or coordinate immunization protocol for
infants, and certain adult subjects) as immunogenic compositions directed against RSV and the PIVs.
Accordingly, certain aspects of the current disclosure provide for broad coverage of an HMPV
immunogenic composition, yielding increased effectiveness against multiple, antigenically-distinct
20   strains. It is also advantageous in certain embodiments to decrease the number of separate viruses that
must be administered: an immunogenic composition comprised of multiple separate viruses is more
difficult to develop and formulate since each virus must be verified to be safe and immunogenic
separately and in combination, and issues such as viral interference can complicate formulation. The
methods of the current disclosure offer a flexibility and versatility in design of immunogenic
25   compositions that addressees these and related problems.

In one exemplary strategy, one or more ORFs each encoding a heterologous, immunogenic
protein or antigenic determinant (for example, fragment or epitope) thereof can be engineered into
transcription cassettes and inserted into the genome of HMPV. For example, as illustrated in FIG.
34, an ORF encoding the G, F or SH surface protein of HMPV strain 75 is engineered to be flanked
30   by GS and GE signals from strain 83, and is inserted into the HMPV strain 83 backbone. This results
in a chimeric virus that contains an additional gene and expresses all of the genes of strain 83 as well
as a surface protein antigen of strain 75. This chimeric virus therefore expresses antigens
representing both HMPV antigenic subgroups. This addresses the need to broaden coverage of
immunogenic compositions, as well as meeting the need to minimize the number of separate viruses
35   that must be included in such compositions. A preferred heterologous strain HMPV gene for
insertion into an HMPV backbone would be the F gene, since F has been directly identified as a
major HMPV neutralization and protective antigen. A second preferred gene is the G gene, since its

encoded protein appears to be highly divergent between HMPV strains, exhibiting only 70% amino acid identity between strain 00-1 and 83 compared to 98% identify for the F protein. Similarly, the SH protein is a third preferred gene for insertion into chimeric virus, since it also is relatively divergent and exhibits 85% sequence identity between strains 00-1 and 83. The example illustrated

5   in FIG. 34 employed the same transcription signals and genome insertion site that was used for expression of GFP. However, the methods of the current disclosure allow other signals or insertion sites to be tested. Also, the Example of FIG. 34 involved an ORF from a heterologous strain of HMPV, but the same strategy can be applied to other heterologous ORFs, such as ones from RSV, HPIV1, 2 or 3, other heterologous viruses or pathogens, and other molecules such as

10   immunomodulatory proteins such as granulocyte-macrophage colony stimulating factor. In this way, an HMPV backbone can be used to create additional viruses bearing the surface proteins of heterologous, non-HMPVs. Chimeric viruses of the current disclosure can then be administered on their own, or can be combined with the original parent or other rHMPV to make a multi-virus or multivalent immunogenic composition.

15   **Example 8**

                        Methods of Screening for Compounds that Inhibit HMPV

              The current disclosure demonstrates that expression of the N, P, L and M2-1 proteins alone are sufficient to direct HMPV transcription and RNA replication. This was documented in two ways. First, expression of these proteins was sufficient to direct transcription and RNA replication of a

20   cDNA-encoded mini-replicon derived from the HMPV genome and carrying a reporter gene. Second, expression of these proteins was sufficient to launch the rescue of complete infectious HMPV from a cDNA-encoded antigenome. The antigenome is by definition the complement of the genome and is not able to serve as the direct template for transcription. Hence, both RNA replication and transcription must have occurred under the direction of the supplied proteins in order for a productive

25   infection to be launched, indicating that N, P, L, and M2-1 are sufficient to direct all phases of HMPV RNA synthesis. Therefore, the current disclosure provides novel and readily practiced methods for screening, identifying, designing, and characterizing compounds that inhibit one or more activities of HMPV (for example, transcription, translation, growth, infectivity, virulence, immunogenicity, etc.)

30   In addition, the current disclosure provides new, facilitated methods of monitoring infection by complete HMPV which provide for the screening, identification and design of antiviral compounds that act at any stage in the HMPV replicative cycle. The current disclosure also provides methods for identifying the viral targets of antiviral compounds, thereby providing for improved design of compounds effective in inhibiting HMPV.

35   Potential antiviral compounds effective against HMPV include monoclonal or polyclonal antibodies obtained by biological or recombinant methods, peptides or protein fragments (Lambert *et al.*, Proc. Natl. Acad. Sci. USA 93:2186-2191, 1996), sugar derivatives, polyionic polymers, anti-

sense RNA, double stranded RNAs capable of meditating RNA interference RNA (RNAi) (Bitko and Barik, BMC Mirco. 1:34-44, 2001), and small molecule compounds (De Clercq, Antimicrob. Agents 7:193-202, 1996; Prince, Ex. Opin. Invest. Drugs 10:197-308, 2002). In general, the methods will apply to any antiviral compounds capable of inhibiting HMPV in solution, in cell extracts, in cell

5   culture, and *in vivo*. Antiviral compounds can act, for example, by inhibiting attachment, penetration (fusion), RNA synthesis, and virion assembly, and in other cases their specific target of action may not be fully elucidated (De Clercq, Antimicrob. Agents 7:193-202, 1996; Prince, Ex. Opin. Invest. Drugs 10:197-308, 2002). Antiviral agents also can act by enhancing the host immune response, such as by stimulating the interferon-mediated antiviral state (Player *et al.*, Proc. Natl. Acad. Sci. USA

10   95:8874-9, 1998). In addition, an antiviral agent can be combined with an anti-inflammatory agent to effect a further reduction in disease (Prince *et al.*, J. Infect. Dis. 182:1326-1330, 2000).

In one embodiment of the current disclosure, rHMPV-GFP is used to screen antiviral compounds. In one exemplary strategy, rHMPV-GFP is treated with a test compound or library of compounds (for example, a compound or library of compounds prospectively including one or more

15   antiviral agents) and compared with mock-treated control rHMPV-GFP for the ability to infect cells. Following a period of absorption, the inoculum containing the potential antiviral compound can be removed by washing if desired. Infectivity is typically assayed by observation or measurement of a detectable label or signal whose presence or level of expression or detection is correlated with one or more activities of HMPV, for example as indicated by the development of green fluorescence due to

20   expression of the GFP marker gene by recombinant HMPV-GFP, a method that can be monitored in living cells without compromising sterility over the course of the infection. Alternatively, the antiviral agent can be included in the medium overlay for the duration of the infection. Alternatively, infection can be initiated in the absence of the compound, which can be subsequently added. In addition to identifying compounds capable of inhibiting one or more activities of HMPV, these

25   assays can give an indication of the mode of action of a compound, such as whether it acts by directly neutralizing virus, or inhibiting subsequent gene expression, or inhibiting viral spread. While GFP is provided as an exemplary label or marker, a wide range of useful "reporter sequences" (for example, reporter genes) and other sequences that direct expression of detectable labels and markers are known in the art and can be readily employed as detectable "signal" agents whose presence, level of activity

30   or expression correlate with one or more selected activities of HMPV. Exemplary alternative markers and/or labels in this context include, *inter alia*, chloramphenicol acetyl transferase, luciferase, secreted alkaline phosphatase, and a large number of other known reporter sequences routinely used in the art. This provides the ability to design a reporter virus or subviral construct that will be useful in a variety of high throughput screening protocols.

35   Within related aspects of the current disclosure, the availability of rHMPV variants that each lack one or more of the viral ORFs, as described above, provides particularly useful tools and methods to identify possible viral targets of antiviral compounds. For example, if a compound

inhibits the infectivity of complete HMPV, it will be tested (for example, for its efficacy and/or target

gene or protein specificity) against a panel of rHMPV lacking various genes, such as the ΔSH, ΔG,

ΔSH/G, and ΔM2-2 variants mentioned previously. An alternative and complementary method of

identifying targets for an antiviral compound involves forcing the virus to grow in the presence of the

5   compound (present at minimal or suboptimal inhibitory concentrations if necessary). Under these

conditions, it is expected that a large panel of useful variants will emerge that have developed

increased resistance to the subject inhibitory compound. Sequence analysis of such variants can

identify mutations that are potentially responsible for the increased resistance. By the methods of this

current disclosure, mutations suspected of conferring increased resistance can be introduced

10   individually or in combinations into rHMPV, and the resistant rHMPV expressing a convenient

reporter gene such as GFP will provide yet additional tools and methods to facilitate other phases of

HMPV control and antiviral development. For example, the introduction of mutations identified in

resistant viruses provides a method of directly confirming that they confer resistance and thus

distinguishes them from adventitious mutation that occur during passage. This provides direct

15   identification of viral genes involved in drug resistance, and is an important tool for further drug

development. As another example, as noted above, HMPV-GFP can be used to assay quickly and

accurately to detect antibodies to HMPV. This can be advantageous in studies to develop monoclonal

or polyclonal antibodies capable of neutralizing HMPV as immunoprophylaxic or therapeutic agents.

This also has applications in epidemiologic studies for example to determine the serostatus of

20   individuals in a population. Furthermore, it provides a facilitated method to monitor changes in the

level of HMPV antibodies in experimental animals or clinical subjects, such as in response to

experimental immunogenic compositions, or in situations were the efficacy of an antiviral compound

is being evaluated. Also, the expression of a reporter gene such as GFP provides a useful marker to

detect and quantify HMPV infection and immunity in individuals, including experimental animals,

25   thereby providing an assay for viral disease detection, tropism and/or to identify and evaluate

additional antiviral agents.

        In other embodiments of the current disclosure, rHMPV expressing a convenient reporter

gene can be readily employed within screening compositions and methods based on cell-free *in vitro*

assays. For example, a nucleocapsid preparation can be made from cells infected with rHMPV

30   expressing a reporter gene, such as alkaline phosphatase or some other sensitively-detected enzyme

or marker. Replicate *in vitro* assays can be constructed and used to screen compounds for antiviral

activity in a high throughput format designed to detect the expressed reporter gene. This can be

based on the expression of a convenient reporter gene or, alternatively, can be based on the detection

of RNA representing the marker gene or one or more HMPV genes. For example, mRNA produced

35   in such an *in vitro* reaction mixture can be captured *in situ* by matrix-bound oligodT and, following

washing to remove the reaction mixture, the resulting hybrids can be detected by secondary

hybridization with probes tagged with reporter enzymes or ligands. Such assays can be further

modified to specifically target, for example, products of RNA replication or read-through of gene junctions as a measure of altered transcription.

The identification of the N, P, L, and optionally, M2-1 as proteins useful and/or sufficient to direct HMPV transcription and RNA replication provides additional new methods for screening compounds for anti-viral activity. In one embodiment, these four proteins can be expressed intracellularly together with a mini-replicon that encodes a reporter gene, resulting in reconstituted HMPV transcription and RNA. This can be used to screen for compounds, particularly those that are effective against HMPV transcription, RNA replication, and nucleocapsid assembly and function. Alternatively, the N, P, L, and M2-1 proteins can be expressed at high levels, using recombinant baculovirus, vaccinia virus, bacteria or any other suitable expression system, and purified to use in reconstituted assays such as *in vitro* RNA synthesis, protein-protein interactions, or protein-RNA interactions. Alternatively, the N, P, L and M2-1 proteins can be tagged recombinantly by the incorporation of, for example, a hexa-Histindine tag or an epitope recognized by a monoclonal antibody, such as the well known FLAG tag (Nilsson *et al.*, Prot. Exp. Pur. 11:1-16, 1997). These provide the basis for purification of these components by affinity chromatography, facilitating the development of *in vitro* assays. In addition, when such tagged genes are expressed from the context of infection by recombinant HMPV, it provides a method of isolating and identifying possible cellular and viral binding partners. Such binding partners can be identified by a variety of methods. For example, in some cases, binding partners will be present in sufficient quantity that they can be visualized by gel electrophoresis and identified by conventional methods of protein sequencing. Low abundance binding partners can be identified from complex mixtures by mass spectrometric methods coupled with trypsin cleavage and protein sequencing. The identification of viral and cellular binding partners provides additional information to guide the development of compounds that inhibit HMPV.

While this disclosure has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations of the preferred embodiments may be used and it is intended that the disclosure may be practiced otherwise than as specifically described herein. Accordingly, this disclosure includes all modifications encompassed within the spirit and scope of the disclosure as defined by the claims below.